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FROM THE DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF HELSINKI

STUDIES ON DETOXICATION MECHANISMS

I

METHOD

by

K. J. V. HARTIALA and SIRKKU RONTU

(Received for publication November 5, 1954)

In connection with previous studies on the mechanism of the experimental cinchophen ulcer it became urgent to have an accurate method for quantitative determination of the glucuronide formation by various tissues. The main requiremnent to be fullfilled by the method was to prove such accuracy so as to allow serial studies under standardized *in vitro* conditions. The method described by Levvy and Storey (2) was chosen for the purpose.

The principle of their method is shortly as follows. Tissue slices are incubated in a special bicarbonate -Ringer solution (1) in the presence of o-aminophenol. The samples are shaken in the Warburg apparatus for a certain period of time after which the tissue slices are removed and dried for later weighing. The proteins are removed from the solution after precipitation with trichloracetic acid and o-phosphoric acid mixture both buffered to pH 2.25. The formed o-aminophenylglucuronide is diazotized and then coupled with naphtylethylenediamine. The formed pink color is measured with a colorimeter.

Since this method has certain special requirements for the purity and quality of the reagents we have studied the possible sources of error in the method before adopting it for routine use. The final procedure as well the modifications made by us will be reported here.

PROCEDURE

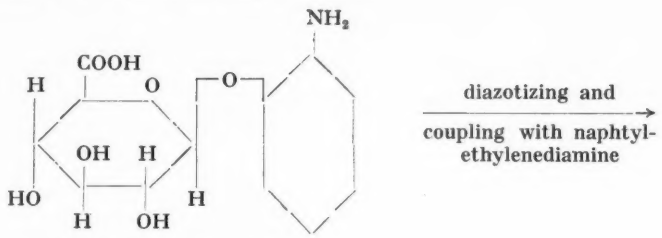
Tissue preparations from the following animal species have been tested: human, cat, dog, rabbit, rat and guinea pig. The small animals were killed by a blow on the head after which the organs to be tested were removed and immediately placed in cold bicarbonate-Ringer. In this solution the magnesium sulphate was replaced by magnesium chloride. This solution was saturated with a gas mixture containing 95 per cent oxygen and 5 per cent carbon dioxide allowed to flow through it from a gas cylinder. The final reaction mixture into which the tissue slices were placed contains in addition to the mentioned Ringer-solution constituents the following substances: 0.2 per cent glucose, 0.17 per cent ascorbic acid, 0.0025 per cent o-aminophenol and 0.224 per cent sodium lactate added in this order. The ascorbic acid and phenol were added as solids.

According to our experience special attention must be given to the purity of the o-aminophenol. The commercial material used by us (Judex) is a dark product which must be purified before use. This appears to be a crucial point in the method. The substance undergoes rapid detoriation which changes the pure colorless crystals into a brown material. If it is necessary to restore it, it should be kept under nitrogen. Regardless of these precautions it is not possible completely to avoid the detoriation for more then about two days. On account of this it is essential to perform the studies with strictly fresh colorless o-aminophenol purified by daily sublimatization and keeping it *dry* under nitrogen. The sublimatization was first carried out in vacuum (7 mm Hg) on an oil-bath at 100°C. The sublimatization under these conditions was rapid but the product was not as good as when obtained by a slower rate without applying vacuum.

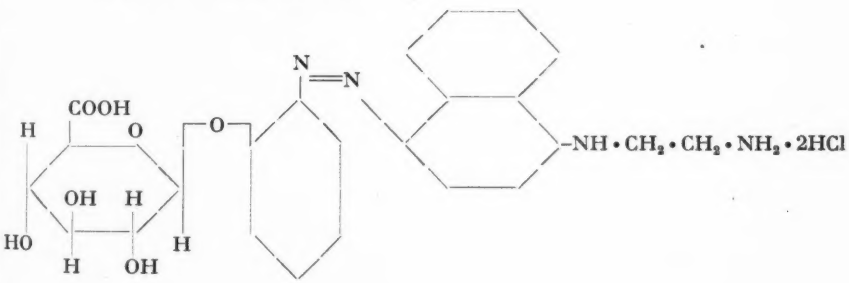
2 ml. of the final Ringer-substrate was delivered into a Warburg vessel. The tissue slices, of size of appr. 10 mg dry weight, were then placed into the flasks. A brisk stream of the oxygen-carbon dioxide gas mixture was then conducted through the manometer and flask system for 1 minute. The incubation took place at a constant temperature of 37°C in the water bath. The respiratory activity could be followed from the manomenter recordings. Incubation period was fixed to 90 minutes. After this the tissue slices were removed and dried at 90°C for 24 hours.

The reaction mixture was deproteonized by adding 2 ml of a mixture containing equal volumes of sodium trichloracetate and sodium orthophosphate to it. The later solutions were prepared daily by mixing equal volumes of stock M-trichloracetate and M-phosphate both adjusted to pH 2.25.

After centrifugation 3 ml of the samples were taken for diazotation. This was carried out as follows. 1 ml of 0.05% $NaNO_2$ solution was first added to the reaction mixture. Several different temperatures were tested in this respect. Obviously the diazonium salt formed from o-aminophenylglucuronide is not as labile as these salts in general so that quantitative results can be obtained also at room temperature. After the nitrite 1 ml of 0.5% ammonium-sulphamate was added in order to bind the excess nitrite. Finally 1 ml of 0.1% naphtylethylenediamine hydrochloride solution was added. The final azo color is formed completely in 2 hours when kept at 25°C. The reactions during the described process may be described by the following schema:



o-aminophenyl-d-glucuronide



The absorption maximum of the pink-violet compound is at 555 mμ (Fig. 1). The intensity of the samples was measured by means of the Beckman spectrophotometer at this wave length. The readings were compared against a blank which contained all

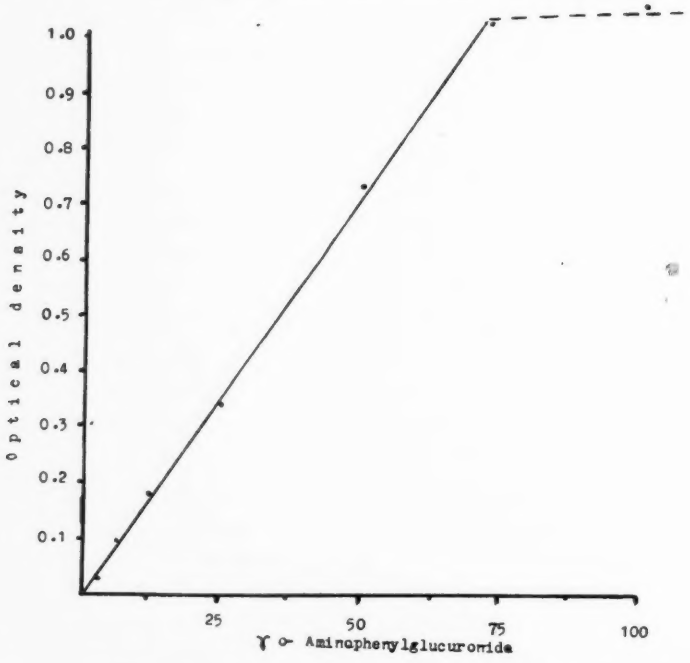


Fig. 1. — Absorption curve of diazotized and coupled o-aminophenylglucuronide. Wavelength 5500 Å.

the used reagents and which were incubated but without any tissue slice.

The *standard curve* was made by first preparing a pure o-aminophenylglucuronide compound by biosynthesis following the instructions given by Williams (3).

A certain amount of pure o-aminophenol is fed to a rabbit by a stomach tube. The urine is collected by a catheter for during the next 15 hours. The glucuronide is first precipitated as a lead salt which is then decomposed with H_2S. After that the filtrate from PbS is concentrated in vacuo at 45—50°C and the glucuronide separated as crystals. The further purification is made with repeated washings with ethanol and ether.

Chemical analyses on the product gave following results

C 48.89% (theoretical value 50.5 %
H_2 5.38% » » 5.30%)

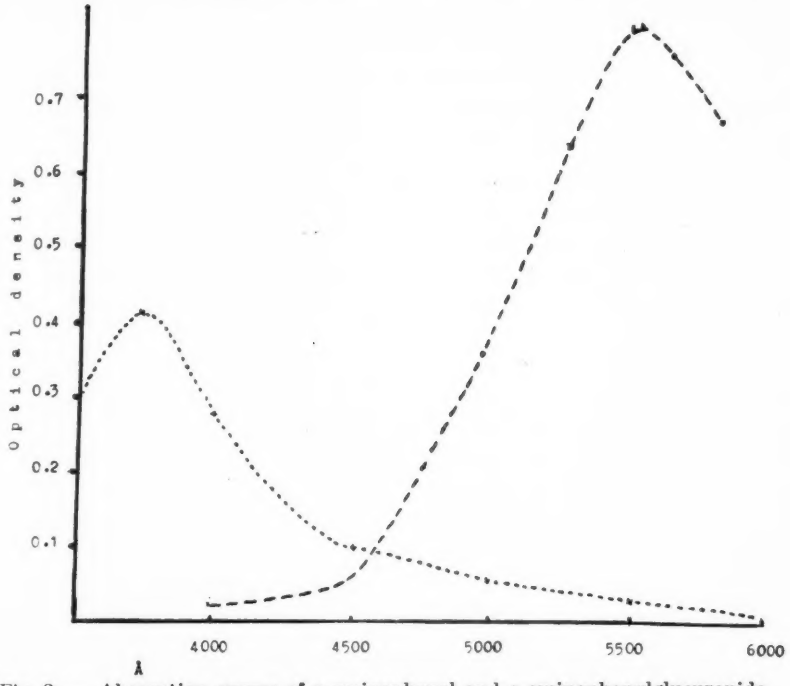


Fig. 2. — Absorption curves of o-aminophenol and o-aminophenylglucuronide diazotized and treated with naphtylethylenediamine.

Obviously this material also undergoes deterioration with time since the chemical analyses performed three months later gave new values. At this time it also failed to give the usual diazocolor.

The calibration curve was made with the fresh material after diazotising it as previously described. From the curve it appeared that quantitative measurements were possible with concentrations of 3 γ o-aminophenylglucuronide per 3 ml of the reaction mixture.

Under the experimental conditions there is always both o-aminophenol and o-aminophenylglucuronide in the reaction mixture. The effect of simultaneous presence of uncoupled o-aminophenol on the results was studied. Various concentrations of both o-aminophenol and o-aminophenylglucuronide were used. Figure 2 illustrates the absorption curves for both compounds. The interference of unconjugated phenol is greatest when only small amount of it has

been coupled. The error becomes progressively less when more glucuronate is formed. According to our analyses the lowest limit for the reliability of the results is with spectophotometric readings above 0.050 corresponding to 47.6 γ o-aminophenol and 6.25 γ o-aminophenylglucuronide in the mixture.

Under these conditions this method renders itself for serial studies. Double determinations made from 20 liver specimens of the same number of rats gave following results: 91 ± 23 γ of o-aminophenylglucuronide per 100 mg dry weight conjugated during 90 minutes.

SUMMARY

The procedure is described of the method originated by Levvy and Storey for *in vitro* determination of the glucuronide conjugation capacity of tissue slices. With special precautions which have been pointed out in this paper this method renders itself for quantitative and serial studies of the detoxication mechanisms.

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FROM THE DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF HELSINKI

STUDIES ON DETOXICATION MECHANISMS

II

EFFECT OF CINCHOPHEN ON THE GLUCURONIDE SYNTHESIS BY THE LIVER[1]

by

K. J. V. HARTIALA and L. TELIVUO

(Received for publication October 22, 1954)

It has long been known that cinchophen in addition to its damaging effect on the liver also exerts a strong ulcerogenic action in certain animal species (3). In connection with studies on the mechanism underlying the production of these pyloric and duodenal ulcers attention has been turned towards the glucuronic acid conjugation mechanism. It is known that several substances which are detoxified in the organism by means of the glucuronide synthesis mechanism also give rise to gastric ulceration (5, 7, 18, 19).

According to the classical views this conjugation takes place in the liver and to a lesser extent in the kidney. Although the fate of cinchophen (2-phenylcinchoninic acid) in the organism is not quite clear some piece of evidence supports the claim that it is also excreted in conjunction with glucuronic acid (17).

The possible relationship of liver damages and gastric ulceration has received both clinical (13, 16, 21) and experimental (1, 2, 6, 11, 18, 20) attention. As specifically regards to the hepatic damage produced by cinchophen it has been pointed out to be a rather selective one since e.g. the bilirubin clearance or serum phosphatase in dogs developing ulcer are not changed (4).

[1] Aided by a grant from the Emil Aaltonen Foundation.

The idea that cinchophen like other similar ulcerogenic agents leads to possible depletion of glucuronic acid has been advanced although the experimental evidence at hand does not furnish any support for the claim. Recently Magee et al. (17) have observed that feeding of cinchophen and para-aminobenzoic acid both increased the urinary glucuronic acid output in dog and rabbit. Only cinchophen of these two agents is known to be ulcerogenic and in these experiments the dog still excreted high amounts of glucuronic acid at the time ulcer had developed.

As mentioned above the main if not only site of glucuronic acid and glucuronide detoxication synthesis is the liver. Previous studies on the glucuronic acid production in cinchophen toxicated animals have been performed on whole animals. In order to specifically study the glucuronide detoxication mechanism of various organs a method was adapted which allows to use surviving tissue slices *in vitro* studies. The agent which is coupled with glucuronic acid is o-aminophenol. This method has been originated by Levvy and Storey (15) and described in detail in a previous paper (10).

MATERIAL

Both guinea pigs and dogs were used in the study. Dog is known to be very suspectible for cinchophen ulceration whereas it has been very difficult or impossible to produce ulcers in guinea pig by this means. According to Schwartz and Simonds (22) these animals succumbed to the toxic effects of the drug without developing ulcers.

Altogether 27 guinea pigs and 5 dogs were used. Cinchophen was given to the guinea pigs in gelatine capsules, the daily dose being 200 mg per kg body weight. Same, ulcerogenic amount was also given to the dogs but with a stomach tube in an aqueous solution of sodium cinchophen prepared by dissolving cinchophen with 5 per cent sodium bicarbonate solution.

The total treatment with cinchophen lasted from 6 to 16 days for the guinea pigs and 10 days for the dogs. This treatment is considerably longer than that reported by Magee et al. in their experiments.

The guinea pigs were killed by a blow on the head. In the dog the liver specimens were rapidly removed under light nembutal anesthesia.

RESULTS AND CONCLUSIONS

The results are listed in table 1. From this table it can be seen that in guinea pig the cinchophen treatment had no effect on the ability of the liver to still produce glucuronides *in vitro*. The cinchophen treatment was rather heavy, many of the animals were tested when they were almost succumbing from the toxic doses of the drug. 4 animals died before it was possible to use them.

TABLE 1

EFFECT OF FEEDING CINCHOPHEN (200 MG/KG × 10 DAYS) ON THE LIVER GLUCURONIDE SYNTHESIS IN GUINEA PIGS AND DOGS. THE RESULTS EXPRESSED AS γ O-AMINOPHENOL/MG DRY WEIGHT

Control		Cinchophen-Treated	
Guinea pigs 8	Mean 124 Range 69—269	Guinea pigs 13	Mean 172 Range 40—387
Dogs 3	Mean 40 Range 16—89	Dogs 2	Mean 80 Range 50—110

As to the dogs the number of animals available was limited. They allow, however, one conclusion to be drawn: the liver of an animal having an ulcer developed by cinchophen still is capable to form glucuronides by its liver and thus participate in the detoxication process. It is especially interesting that at the time of the liver producing 110 γ glucuronide per 100 mg dry weight one dog showed at autopsy 3 ulcers developed during the cinchophen treatment, all these ulcers were pyloric and of considerable size (diameters 12, 7 and 5 mm).

It would therefore appear that the liver damage produced by cinchophen does not involve the ability of this organ to produce glucuronic acid and form conjugated glucuronides neither in an ulcer suspectible animal (dog) nor in a species (guinea pig) in which cinchophen ulceration does not occur.

The depletion of the liver glucuronic acid sources can not in light of these and some earlier observations (17) be kept responsible for the heavy reduction of the glucuronide-containing mucus secretion by the pyloric and duodenal glands (8, 9, 14) and does not explain the mechanism underlying the production of cinchophen ulcer.

SUMMARY

The ability to form glucuronides by liver slices taken from cinchophen toxicated guinea pigs and dogs was studied by means of measuring the conjugation power for o-aminophenol.

It appeared that in both species the liver continued the synthesis of glucuronides with the original rate. No ulcers were observed in guinea pigs.

In the dog the cinchophen treatment resulted in the usual ulcer formation. Liver specimens taken at the time ulcers had developed showed still a very good capacity to form glucuronides.

It was concluded that the depletion of the liver sources of glucuronic acid as well as the failure of the liver to perform its detoxication activity does not explain the mechanism underlying the production of cinchophen ulcer in dog.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI

EFFECT OF PSYCHIC STRESS ON THE HEART OF THE RAT

by

ESKO K. NÄÄTÄNEN

(Received for publication October 26, 1954)

Repeated efforts have been made in recent years to discover the reason for the growing incidence of myocardial infarction in many countries. One of the reasons considered is the hurry and mental stress characteristic of life today. In order to ascertain the influence of mental stress on the etiology of coronary sclerosis and myocardial infarction, the prevalence of these diseases in different occupations has been investigated. Certain researchers (4, 7) regard it as having been established that the said diseases are commonest in those occupations where practitioners are exposed to most mental stress (as physicians and business men). On the other hand, there are a considerable number of investigators who have reached the opposite conclusion (2, 6). In his handbook on pathology of the heart, S. E. Gould (3) asserts that the occupation of the patient evidently has little bearing on the etiology of coronary sclerosis.

Any protracted serious disease gradually leading to death is doubtless accompanied by heavy mental stress. It would therefore seem reasonable to assume that coronary sclerosis and myocardial infarction occur more often than usual among such patients, but, according to Wilens, among cancer and tuberculosis cases these diseases are rarer than is usual.

Inasmuch as the views of different investigators conflict so much in regard to the effect of mental stress on the etiology of the heart diseases mentioned, it would seem quite necessary to try to throw light on the question by means of experiments on animals. Loud noises repeated at irregular intervals and flashes of light have struck me as the best ways of provoking stress in laboratory animals. My aim has been to excite rage and fear in them and then study the changes in the heart caused by such stress factors applied over long periods of time.

MATERIAL AND METHOD

The experiments were performed on 15 male rats ranging in weight from 150 to 200 g. They were exposed to irritants for several hours every day for periods varying between 12 and 105 days. The rats heard different kinds of loud noises repeated at varying intervals, and they were kept during the period of experiments in a dark room lit from time to time by a bright lamp. The animals responded to these irritants in different ways; some tried to hide in a corner of their cage, some became enraged and began to fight with their fellows. One, bigger than the rest, killed four others and ate them in part, notwithstanding the fact that plenty of ordinary nourishment was fed the rats.

Treatment having been given sufficiently long, the test animals were killed by decapitation after they had been stunned by a light blow. The hearts were removed, weighed and then fixed in 10% formaline and embedded in paraffin in the usual manner. Haematoxylin-eosin was chiefly used for staining.

RESULTS

Macroscopic Examination. — In the rats exposed to stress the area of the apex of the heart was rounded and the heart appeared enlarged. The weight of the organ ranged from 690 mg to 356 mg per 100 g of body weight; the mean was 460 mg/100 g. The corresponding figures for the control animals were smaller: 392—280 mg/100 g, the mean being 348 mg/100 g.

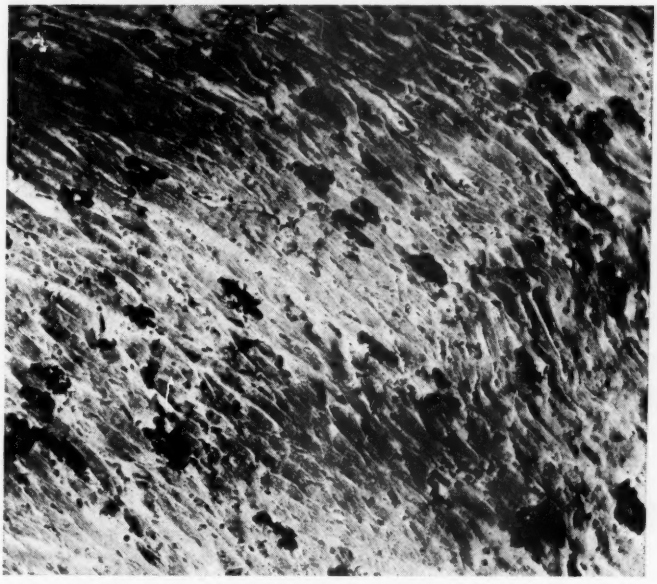

Fig. 1. — Photomicrograph showing a section of the heart of a rat exposed to stress for 39 days. Muscle fibers largely degenerated, nuclei generally disappeared. Abundant accumulations of pigment. × 200.

Microscopic Examination. — In the heart of each rat exposed to stress surprisingly great changes occurred. Generally hypertrophic fibers could be observed, but in addition there appeared areas in which the tissue had undergone different degrees of degenerative change. In the degenerated areas the striation of the fibers had usually disappeared. There was coagulation of the sarcoplasm into a homogeneous mass together with shrinking of the nuclei. In some places the nuclei had totally disintegrated; in such areas there could often be seen accumulations of pigment crystals (Fig. 1). In several preparations the muscle fibers were swollen in places (Fig. 3) and there was a tendency for vacuoles of different sizes to occur. In other preparations, again, the muscle fibers were observed to be atrophied in places and to exhibit fragmentation (Fig. 4). In cases where the rat had lived for a long time after damage to the tissue, the local connective tissue cells were seen to have proliferated and the necrotic focus to have been replaced by fibrous tissue.

Fig. 2. — Photomicrograph showing a section of the heart of a rat exposed to stress for 105 days. In the artery, whose wall is considerably degenerated, we observe a thrombus with an abundance of cells containing pigment. Connective tissue growing into thrombus. × 90.

In the rat that had lived under stress for 105 days, macroscopic examination revealed that the apex of the heart was covered with scar tissue over a fairly extensive area. Under the microscope a coronary thrombosis was revealed (Fig. 2).

Quite marked changes in the arterioles of the heart wall could be detected in all the test animals. Often there appeared subintimal deposition of hyaline material. Longitudinal sections showed that this material was deposited along the vessel wall in masses of unequal thickness. The lumen of the vessel was in many cases greatly reduced in size, and sometimes occluded. Medial hypertrophy, together with degeneration of collagen, was often noted. Degeneration of collagen was accompanied in the muscle cells by different degenerative changes, shrinkage, vacuolisation and pycnotic nuclei. Where the degenerative changes were considerable, the vessel wall had evidently become fragile and liable to disintegrate easily.

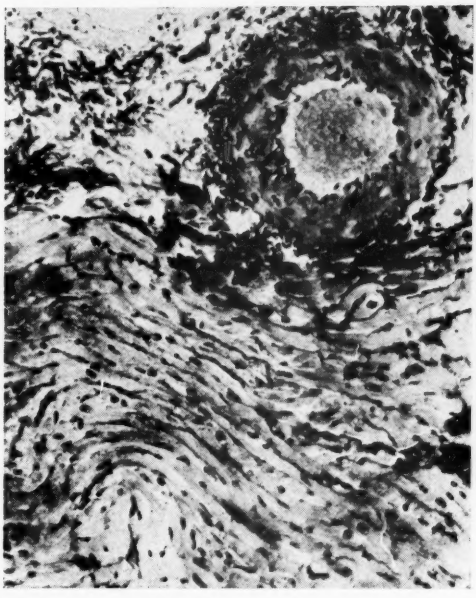

Fig. 3. — Photomicrograph showing a section of the heart of a rat exposed to stress for 12 days. Hydropic degeneration of muscle fibers. Hyaline degeneration in the wall of the vessel. × 200.

DISCUSSION

The changes observed in the hearts of rats exposed to stress proved to be amazingly great. No clear correlation between the length of the period of stress and the extent of the changes in the heart could be established. This apparently is a consequence of individual differences. During the course of the experiments it was observed that the rats reacted under stress in very different ways. Some tried to hide, while others put up a fierce resistance, fighting among themselves when they were unable to engage an external enemy.

It is a generally known fact that mental stress commonly raises blood pressure and accelerates the action of the heart. The increased strain on the organ thus caused, provided it has lasted sufficiently long, probably accounts for the hypertrophy of the heart observed in the test animals.

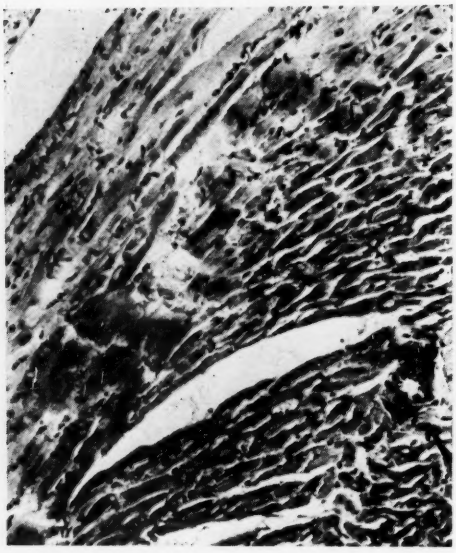

Fig. 4. — Photomicrograph showing a section of the heart of a rat exposed to stress for 23 days. Atrophic, fragmented fibers. Colour of muscle tissue in places darker. Note the arteriole with thickened wall. Cell infiltration around vessel. × 200.

In the same way it may be conceived that protracted mental stress could be the cause of hypertrophy of the human heart in cases where the etiology is otherwise inexplicable. This is liable to happen especially to sensitive persons, who become emotionally agitated under even slight stress.

Serious lesions could be noted in the hearts of all the rats exposed to stress. How is it to be explained that psychic stress can lead to a coronary thrombosis and necrosis of the myocardium?

Degenerative changes have been experimentally produced in the media, namely hyaline and fatty degeneration, followed by necrosis, by injecting large doses of adrenalin. It is apparent that the result is brought about by spasm of the vessels. Prolonged spasm causes anoxemia of the muscular coat, followed by degeneration and necrosis. A strong prolonged secretion of adrenalin during the period of stress could perhaps be partly responsible for the lesions observed in the vessels. Changes occurring in the vessel wall often cause narrowing or obliteration of the lumen of the vessel. Thereupon that part of the heart muscle fed with blood

by the vessel no longer receives nourishment, inducing an ischaemic condition and consequent degeneration of the tissue.

The observations presented in the foregoing cannot, of course, be applied offhand to human beings. Nevertheless, it would appear that mental stress is frequently a more important factor in the etiology of serious blood vessel changes and myocardial infarction than has hitherto been supposed. This would seem to apply especially in the case of persons sensitive to psychic irritants. Observations made in connection with obductions tend to support this view. Numerous researchers have noted that myocardial infarction can occur also in cases where there has been no coronary occlusion and where not even the coronaries showed any intimal changes or narrowing of the lumina (1, 8). The cause of myocardial infarction in such cases could be an intense vasospasm brought on by mental stress.

SUMMARY

For the purpose of studying the effect of psychic stress on the heart, fifteen male rats weighing between 150 and 200 grams were exposed to stress for periods ranging from 12 to 105 days. Loud noises and flashes of light were used as irritants to produce stress.

1) Stress appeared to affect the general condition of the animals strongly, causing a lowering of their resistance.

2) In the hearts of the test animals there could be observed hypertrophy, various degrees of degeneration and necroses as well as, in some cases, the formation of a thrombus. In the walls of the arterioles hyaline degeneration had taken place; the walls had often grown thick and the lumen of the vessel become narrowed or occluded.

3) No clear correlation between changes in the heart and the length of the period of stress could be established. The reason probably is that the rats reacted to stress as individuals in different ways.

4) The primary cause of myocardial changes was believed to be prolonged vasospasms interfering with the nourishment of the tissue. Vasospasms conceivably also cause morphologic changes gradually appearing in the vessel wall.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI

EFFECT OF INTENSE MENTAL STRAIN ON THE MORPHOLOGICAL PICTURE OF THE TESTES

by

ERKKI O. JÄNKÄLÄ and ESKO K. NÄÄTÄNEN

(Received for publication October 23, 1954)

It has often been noted in practice that severe prolonged mental strain is associated with disturbances of the libido, potency and fertility, which probably are phenomena resulting from functional disorders of the sexual glands. In debility, schizophrenia and diseases of the hypothalamus there occurs acquired atrophy.

A considerable loss of weight has been noted in the testes of criminals awaiting sentence of death, likewise degenerative changes in the seminiferous epithelium, loss of the mature spermatozoa, and a reduction in the diameter of the tubuli contorti. On the other hand, changes have not been observed in the interstitial tissue (5).

Intense degeneration of the seminiferous epithelium with formation of polynuclear giant spermatocytes has been found in men who had committed suicide or had been executed (4). We found no references in the literature concerning the effect of psychic stress on the morphology of the testes of animals.

MATERIAL AND METHOD

The experiments were performed on 20 male rats, the length of the experiment time ranging from ten to 105 days. The mental

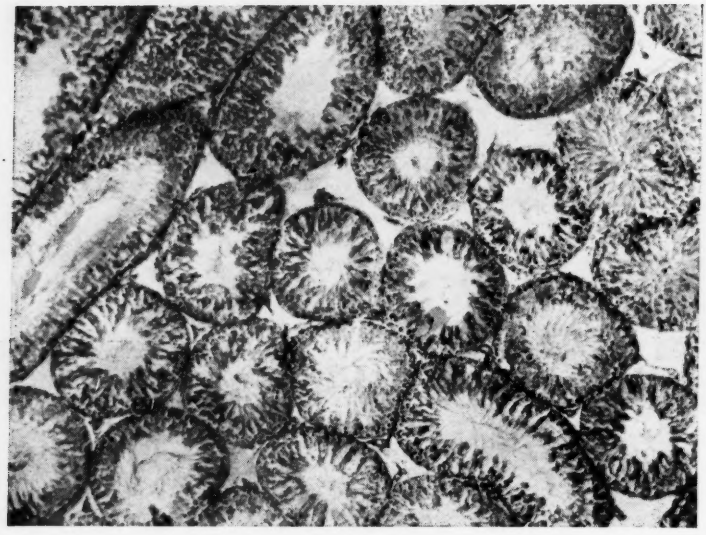

Fig. 1. — Stress 41 days. Early degeneration. Spermatids and prespermatids have disappeared in many tubuli. Degenerative changes can also be seen in spermatocytes and in spermatogones. At the upper right corner filled tubuli. × 100.

stress was produced by irritating the animals for several hours a day by means of bright flashes and sudden loud noises, repeated at irregular intervals, as well as, in the case of one group, simultaneous immobilization. The animals became very quarrelsome and furious, and it occurred a few times that some of them even killed each other.

By the end of one month some of the rats began to show signs of fatigue, such as tremor, unbalanced walk and involuntary muscular twitchings. A few rats died without any apparent cause.

Individual differences in the behavior of the rats were very conspicuous. During the treatment, some became very aggressive, even furious, and often threw themselves on their backs, while others responded to the treatment more passively and shied away.

In our opinion, the stress was so severe that the animals were not capable of adapting themselves to it and could not, accordingly,

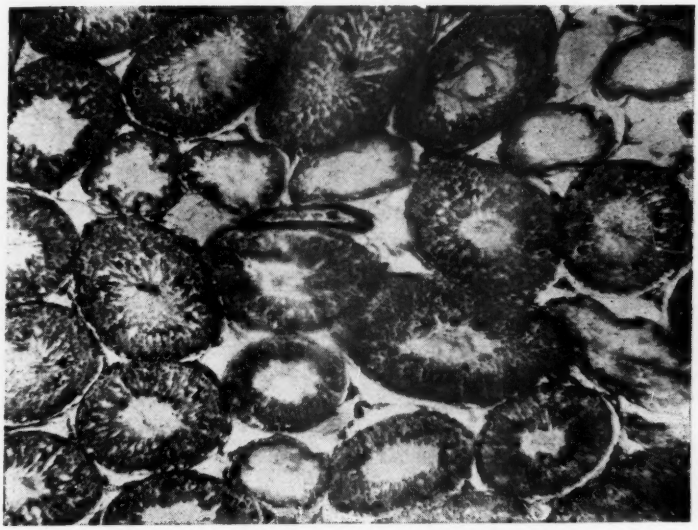

Fig. 2. — Stress 30 days. Degeneration of various degrees. A few Sertoli's cells and spermatogones can be seen in some atrophic tubuli. All cells of the seminiferous epithelium occur in some tubuli and partial filling of the lumen can be observed in some. × 100.

reach the stage of resistance. The animals were killed after stress periods of varying length. The testes were fixed in formalin and stained with hematoxylin-eosin.

RESULTS

The degree of the morphological changes was not directly proportional to the time, but notable differences occurred between the individuals which were exposed to similar stress for equally long periods. After four weeks, the testes of two rats became hypertrophic and the tubuli contorti were filled with spermatids. In three other rats, on the other hand, the weight of the testes was lower than normal. Vacuolization was observed in the interstitial tissue. In many tubuli the cell debris had filled the lumen, and cytolysis occurred in the cells of the seminiferous epithelium, which were swollen and poorly stainable, and often had pycnotic nuclei. Both the spermatids and prespermatids had disappeared in many tubuli.

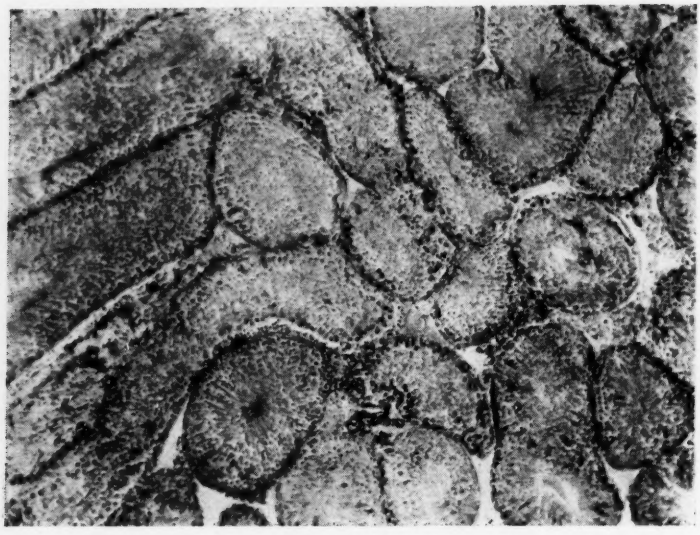

Fig. 3. — Stress 30 days. Cell debris has filled the lumen of the tubuli. Degenerative changes occur in all cells of the seminiferous epithelium. The cells are swollen, poorly stainable, and their nuclei are often pycnotic. × 100.

After six weeks, atrophic changes were noted in the testes of all the animals, the average weight being about two-thirds of normal. It was conspicuous that the degenerative changes in the different tubuli of the same testis frequently varied. *E.g.*, in some tubuli only a few Sertoli's cells and spermiogonia occurred, in some spermiocytes in addition, and in some all cells of the seminiferous epithelium. In others, however, acidophilic cell debris had filled the lumen and degeneration occurred in the cells of the seminiferous epithelium to a varying extent. Besides, in the same rat the weight of one testis might be but a half that of the other, the degenerative changes varying correspondingly.

After three months' intense mental stress, total atrophy of the seminiferous epithelium could be noted, the weight of the testes ranging from one-fifth to one-half of normal. In most tubuli Sertoli's cells and the cells of the seminiferous epithelium had entirely disappeared and in some tubuli only a few Sertoli's cells and spermatogones were noted. A rich local connective tissue proliferation was found in the interstitial tissue and considerable

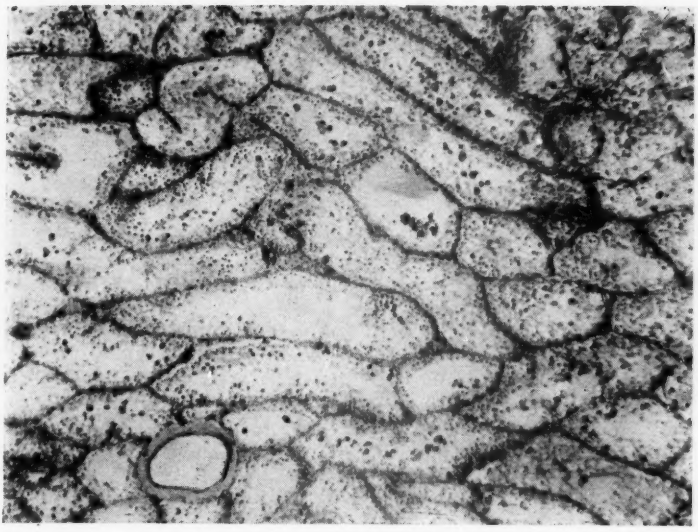

Fig. 4. — Stress 46 days. The cell debris filling the lumen is dissolving. Loose degenerative cells of various degrees can be seen in the lumen. × 100.

hyaline degeneration appeared in the wall of the arterioles of the atrophic testes. The wall of the arterioles was thickened and the lumen narrowed, often almost entirely occluded.

DISCUSSION

It is generally assumed that the testis atrophy resulting from prolonged mental strain is due to a diminution of gonadotrophin production under conditions of stress, which call for an increased elaboration of adrenotrophic and perhaps also other metabolism-influencing anterior-lobe hormones.

The administration of anterior pituitary gonadotrophins restores the testes even in hypophysectomized animals. FSH is necessary for the maintenance of the spermatogenic epithelium, and LH for the structural integrity and endocrine function of the Leydig cells. Suitable mixtures of FSH and LH prevent degeneration in the testes of rats exposed to a variety of systemic stresses.

Abnormal, or even extreme, temperature has been used as a stress agent in most experimental studies of the morphology of the testes associated with the stress syndrome. The changes

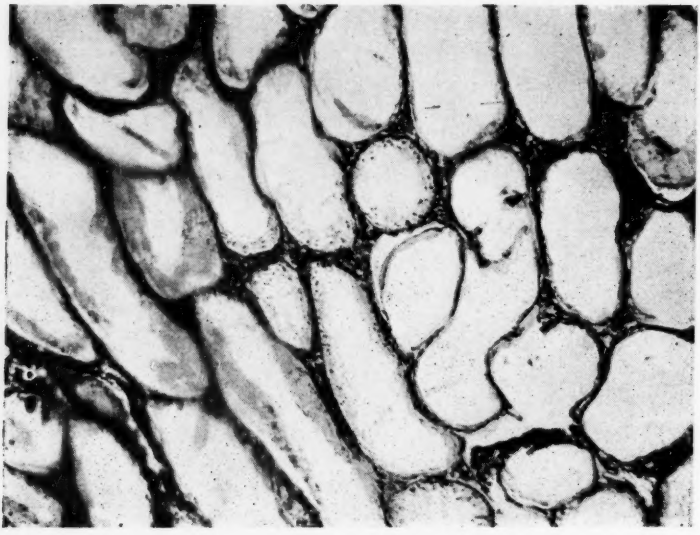

Fig. 5. — Intense stress 105 days. A few spermiogonia and Sertoli's cells can be seen in some tubuli. Connective tissue proliferation occurs between the tubuli. × 100.

have been typical by their nature and seemed to affect the spermatids first, the spermatocytes second, and the spermatogonia last. The interstitial cells showed only a slight swelling with some vacuolar degeneration (1, 6). In some cases, attempts have been made to interpret the changes in the testes caused by exceptional temperatures as results of systemic stress in accordance with the general adaptation syndrome (3, 6).

In our opinion, however, abnormal temperature specifically prevents spermatogenesis, and atrophy results. This concept is supported by the fact that a general or local temperature change of a few degrees only, such as artificial cryptorchism, produces involution of the seminiferous epithelium. The involution may also be caused by a special heating apparatus placed around the scrotum, although this intervention could hardly have caused any systemic damage.

The changes caused by systemic mental strain are not linear in the same way, *i.e.*, degenerative changes in all the tubuli of the testes are not equal, as is the case in the changes produced by

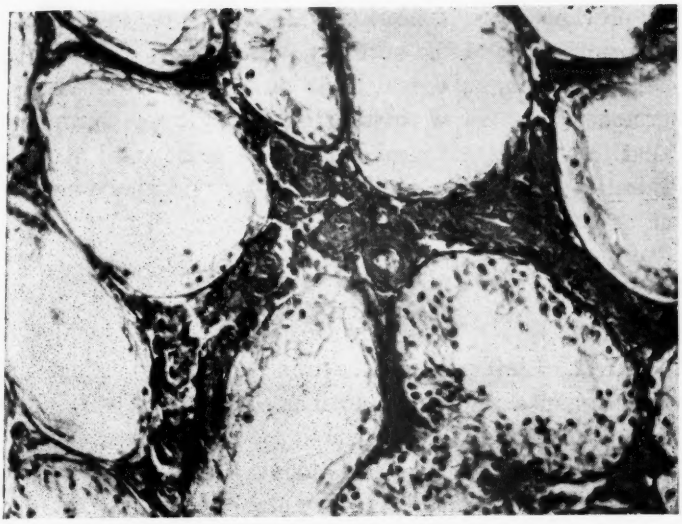

Fig. 6. — Three months' intense stress. The walls of the arterioles of the interstitial tissue of the atrophic testis are thickened and the lumens of some small vessels are almost completely occluded. Hyaline degeneration can be noted in the wall of the blood vessels. × 300.

abnormal temperatures. The effect on the interstitial tissue also differs between them.

We are of the opinion that there exist two entirely different mechanisms: 1) the specific effect on the seminiferous epithelium produced by abnormal temperatures, and 2) the effect of mental strain on a hormonal basis in agreement with the general adaptation theory.

During the experiments the animals lived in total abstinence. This did not, however, influence the results, as the histological picture of the testis remains essentially normal in animals and men who have had no sexual intercourse for very long periods (4).

That the seminiferous epithelium of a tubulus was totally atrophic and that the tubulus next to it remained entirely unchanged could not be explained with certainty. The marked hyaline degeneration of the blood vessels and the total occlusion of the lumen of various arterioles suggested, however, that these changes may be caused by nutritional disturbances. Severe changes in the blood vessels were observed also in other organs.

Our previous work concerning the effect of mental strain on the hypophysis and the adrenals showed that, on the basis of morphological changes, considerable alterations occurred also in the hormonal secretion of these animals (2). It was surprising to note that intense mental strain may even cause total atrophy of the seminiferous epithelium, a fact not observed in previous studies on men or animals.

SUMMARY

The effect of intense mental strain on the morphology of the testes was studied on 20 albino rats. The stress, varying from one to three months in length, caused severe degenerative changes, even total atrophy of the seminiferous epithelium. Individual differences were, however, quite notable. The degree of the degeneration varied largely in the different tubuli of the same testis. In the atrophic testes considerable hyaline degeneration of the blood vessels and narrowing of the lumen were observed.

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FROM THE DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF HELSINKI

STUDIES ON DETOXICATION MECHANISMS

III

GLUCURONIDE SYNTHESIS OF VARIOUS ORGANS WITH SPECIAL REFERENCE TO THE DETOXIFYING CAPACITY OF THE MUCOUS MEMBRANE OF THE ALIMENTARY CANAL

by

K. J. HARTIALA

(Received for publication November 3, 1954)

Glucuronide synthesis, an important part of the detoxication mechanisms is known to be carried out in the liver and to a lesser extent in the kidney (7, 8, 10, 12, 14, 15, 17). In a course of studies of the mechanism of the experimental cinchophen ulcer the writer became interested on the detoxication mechanisms and specifically on the glucuronide formation. This interest was aroused by the fact that cinchophen leads to increased glucuronide output in the urine when fed to animals and is also obviously detoxicated by this means.

It is known that certain biological products contain glucuronic acid as well as glucuronides. Thus e.g. the synovial fluid and other hyaluronic acid containing compounds have glucuronic acid as a part of their molecule. Mucoprotein products of the salivary glands and of the gastric, pyloric and duodenal glands give a positive reaction for glucuronic acid.

Cinchophen is known to cause a reduction in the mucus secretion by the pyloric and duodenal glands (2, 4, 9) and this fact has been connected with the mechanism underlying the ulcer formation produced by cinchophen. The possible depletion of the liver sources of glucuronic acid has been a previous working hypothesis. It has, however, been shown that the whole organism (12) as well

as liver slices taken from cinchophen toxicated animals (6) still are capable to carry out glucuronide synthesis at the time ulcer has developed.

Having these facts in mind but still assuming that the cinchophen ulcer and glucuronic acid conjugation mechanism have some connection with each other lead the author to perform studies on the glucuronide synthesis by other organs. The main purpose of these experiments was to find out whether the liver really is the only and main site of detoxication or would it be possible that other organs also participate in this important activity, mainly those which also produce glucuronic acid containing material.

These studies were performed using the method advanced by Levvy and Storey (10). The procedure as well as our modification has been described in detail (5).

METHOD AND MATERIAL

Altogether some 200 rats, 23 guinea pigs, 8 cats, 35 rabbits and 5 dogs have been used in this study. The most suitable size for the tissue slices was found to be around 10 mg dry weight. The usual slicing technique for making specimens from the mucous membrane was found to be unsatisfactory. After some experimental trials it was found to be satisfactory to cut them with sharp scissors. The thickness of the slices was tried to make as equal as possible.

The animals were killed by a blow on the head except the dogs in which the organ specimens were taken under a light nembutal anestesia. The human placentar specimens were obtained immediately after the delivery, placed in Ringer solution in an ice-container and brought in some 15 minutes to the laboratory.

RESULTS AND CONCLUSIONS

Table 1 gives the results produced by liver and kidney slices taken from various animal species. All tested animals showed a similar glucuronide conjugation power with the exception of cat. In this animal the liver as well as kidney showed no, or only very little signs of this synthesis. This species difference, if true, is interesting and deserves further investigation.

Results obtained with other organs than liver and kidney are listed in table 2. All these organs gave a negative result.

TABLE 1

GLUCURONIDE SYNTHESIS OF THE LIVER AND KIDNEY IN VARIOUS ANIMAL SPECIES. γ O-AMINOPHENOL CONJUGATED (100 MG DRY WEIGHT OF TISSUE) 90 MINUTES

Organ	Animal	Range	Mean
Liver	Rabbit	38—236	123
	Rat	24—195	65
	Guinea pig	69—269	124
	Dog	40—110	66
	Cat	trace?	—
Kidney	Rabbit	38—138	88
	Rat	24—156	79
	Guinea pig	43—96	80
	Dog		
	Cat	trace?	—

TABLE 2

LIST OF ORGANS TESTED FOR GLUCURONIDE SYNTHESIS

Organ	Animal	Results
Pancreas	Rabbit, rat, cat, guinea pig	negative
Spleen	» » dog, » »	»
Adrenals	» » » » »	»
Ovary	» » » » »	»
Testicle	» »	»
Placenta	» » human	»
Peritoneum	» »	»
Muscle		
diaphragm	» »	»
skeletal	» »	»

TABLE 3

GLUCURONIDE SYNTHESIS. LIST OF ORGANS KNOWN TO CONTAIN OR PRODUCE GLUCURONIC ACID AND TESTED FOR THEIR ABILITY TO CONJUGATE O-AMINOPHENOLGLUCURONIDE IN VITRO

Organ or Tissue	Animal	Result
Synovial membrane	Rabbit, rat	negative
Umbilical cord	Rabbit	»
Excised eye, various parts	Rabbit, rat	»
Trachea, mucous membrane	Rabbit, rat	»
Ureter, » »	Rabbit, rat, guinea pig	»
Urethra, » »	»	»
Urinary bladder, mucous membr.	» rat, dog, cat	trace?

TABLE 4

GLUCURONIDE SYNTHESIS BY THE MUCOUS MEMBRANE OF THE GASTRO-INTESTINAL TRACT. O-AMINOPHENOL CONJUGATED (100 MG DRY WEIGHT TISSUE) 90 MINUTES

Organ	Cat	Dog	Rabbit	Rat
Stomach				
greater curvature	—		10—100	62— 71
lesser curvature	—		25—217	—150
pyloric canal	—	32—145	69—240	30—511
Duodenum	—	55—160	28—568	48—567
Ileum	—	—175	82—328	40—208
Colon	—		29—300	27—657

In the next series those organs were tested which either produce glucuronic acid containing compounds or which otherwise contain it. These results, also negative, are listed in table 3.

The studies were now continued by taking slices from various parts of the alimentary canal beginning from the stomach. The results are listed in table 4. From this table it can be seen that slices taken from the mucous membranes of the stomach, duodenum, ileum and colon all gave markedly high values for glucuronide synthesis. The results were also positive in all other animals tested except in cat.

In order to secure that the positive reactions obtained by the method are really the result of an active glucuronide synthesis several control experiments were performed. The possibility remained that the glucuronic acid itself or glucuronide constituents of the tissue specimens were capable to give a positive color reaction after diazotation and coupling with naphtylethylendiamine under the experimental conditions.

First pure duodenal juice collected from duodenal pouches in dog was treated with the diazo-reagents and naphtyletylendiamine. No positive color reaction occurred.

Thereafter pure duodenal juice was added in varying amounts to the Ringer-solution used in the usual procedure. The diazo and coupling procedures were again carried out. No positive reaction occurred.

Tissue slices were kept over varying periods at room temperature or killed by boiling. Specimens treated so and which gave no signs

of respiratory activity in the Warburg-apparatus gave entirely negative results.

Duodenal mucus is known to undergo a proteolytic hydrolysis by various agents (3). In order to test whether the hydrolysis products of duodenal mucus give positive reactions duodenal juice was incubated with crystallized trypsin at a pH of 7.6 for 1 hour. At this time the thick mucus had changed to a watery-like material. Diazo and color reactions made with this material gave negative results.

All these observations indicate that glucuronide synthesis for detoxication purposes is not confined only to the liver and kidney but takes place along the mucous membrane of the alimentary tract. It would thus appear to take place in regions where the organism first, most and strongest becomes in contact with toxic agents: in the stomach, intestine, liver and kidney. All other organs so far tested have given negative results. These results would also be in line with those obtained with the ethereal sulphate conjugation detoxication. Certain toxic agents are detoxicated by means of this synthesis which has been shown to take place perhaps also in the intestinal wall (1).

In contrast to the reported observations on the sulphate conjugation activity by the intestinal elements our own observations would indicate that the glucuronide synthesis in the intestine is carried on with a great capacity. Although the results would show it being even greater than in the liver we would at the moment be cautious to emphasize such point. The method applied in this study is, according to our experience, reliable as far the specimens of the same tissue is concerned. Liver and mucosal specimens are, however, so much different in their physical appearance that it is impossible by the given method have the slices equal as far as the thickness and surface dimensions are concerned. The values calculated on the dry weight basis may therefore not be comparable for the different types of tissues. This point is now under studies in this laboratory.

As to the original problem of cinchophen ulceration observations that glucuronide synthesis takes place also locally by the mucous membrane of the gastric, pyloric and duodenal regions brings the problem in an entirely new light. The question might not be of a general systematic disorder caused by cinchophen but of a syndrome caused by unfavourable local conditions.

The toxic agents such as cinchophen need not exhaust the total sources of glucuronic acid — which it apparently also does not. It would be sufficient that this agent when coming in contact with the mentioned areas shifts the physiological mucoprotein secretion towards the protective glucuronide-detoxication synthesis. Obviously this occurs along the whole intestinal canal. That the ulcers occur solely on pyloric and duodenal regions might be explained by the fact that these regions are the first to carry the extra stress caused by the gastric hydrochloric acid and pepsin output. Several earlier observations could be explained on this basis. The beneficial effect of feeded mucin preparations on prevention of cinchophen ulcer (13) would be based on the neutralizing effect of the mucin on the gastric contents. Cinchophen ulcers can not be produced in all animal species. In a typical ulcer nonsuspectible species, rabbit, cinchophen causes, however, an increased glucuronide output similar to ulcer suspectible animals (12). In this animal cinchophen also causes a reduction of the protecting mucus secretion by the pyloric glands (12). The mucous membrane of the pylorus and duodenum in rabbit also synthesize glucuronides in vitro, their detoxication mechanism as far the glucuronide conjugation activity is concerned does not differ from an ulcer suspectible animal. In rabbit the effect of HCl and pepsin might, however, not be as strong as in some other, mainly carniforous animals, due to the fact that in rabbit the stomach is constantly full with food. The hydrochloric acid and pepsin is not hence so free to attack the gastric wall. The problem would be the same as for that of producing histamine ulcer in rabbit. Whereas rabbits on regular diet do not develope ulcer even by injecting histamine in wax, ulcers have been claimed to occur following histamine injection by feeding the animals a liquid diet which obviously passes the stomach rapidly and leaves it empty with its gastric secretory products (16).

SUMMARY

The glucuronide conjugation of various tissues and organs of different animal species has been studied *in vitro*.

Most of the organs gave negative results. Among these were the synovial membrane, mucous membranes of the trachea, uterus ureter, urinary bladder. In addition to the liver, specimens taken

from the alimentary canal all gave highly positive results indicating that the glucuronide detoxication mechanism is not only confined to the liver and kidney.

The specific bearing of these observations on the experimental cinchophen ulcer is discussed.

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FROM THE DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF HELSINKI

STUDIES ON DETOXICATION MECHANISMS

GLUCURONIDE SYNTHESIS IN FOETAL RABBIT

IV

by

K. J. V. HARTIALA and M. PULKKINEN

(Received for publication October 3, 1954)

Whereas morphological and histological studies are numerous and have thoroughly explored the time at which various organs develope during foetal life, considerably less attention has been given to study the time at which various organs and tissues first possess their ability to perform their known adult functions.

In connection with some studies of the glucuronide detoxication synthesis we had the opportunity to carry on experiments with pregnant rabbits. The liver of the fetus of these animals were tested for the ability to perform glucuronide detoxication.

MATERIAL AND METHODS

The detoxication synthesis was followed by using o-aminophenol as the toxic substance and measuring the glucuronide formed with it. The procedure has been described previously (1, 2). Altogether 37 rabbits, 34 fetus and 9 newly born rabbits were used. The female rabbits were kept together with the male for 24 hours and the age of the foetus was counted from this time. 7 rabbits out of 15 became pregnant. The fetus were delivered by caesarean section, the livers were then immediately removed and placed into ice-cold Ringer solution. The liver of the mother-animal was treated similarily.

This study has been supported by a research grant from the State Council for Natural Sciences (Valtion Luonnontieteellinen Toimikunta).

Several specimens from the placentar tissue were also subjected for the glucuronides synthesis procedure. In addition data from 30 adult rabbits were collected.

RESULTS

The results obtained with the foetal livers are illustrated by the figure. From these results it appears that the rabbit has the ability to perform glucuronide synthesis at the time of birth although this power perhaps is not in a newly born as great as in the adult rabbit. The average results from 30 adult rabbits gave 123 γ per 100 mg dry weight and the highest value for the newly born rabbit was 52 γ. From these experiments it would also appear that the ability to perform glucuronides begins at the later half of the pregnancy.

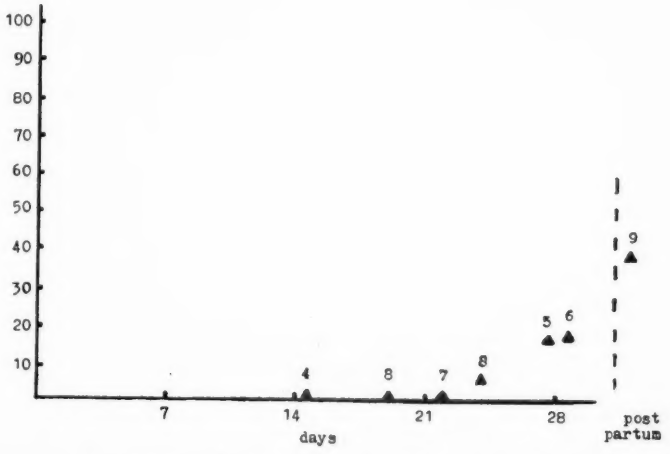

Fig. 1. — Glucuronide synthesis (o-aminophenol) of foetal rabbit. The results expressed as γ o-aminophenol synthesized per 100 mg dry weight liver. The time refers to foetal age. Number of fetus given above the mean values.

Placentar tissue analysed at different phases of the pregnancy always gave negative results for glucuronide synthesis. It would thus appear that the foetus is in earlier phases of its life entirely dependant on its mother-animal as far as the glucuronide detoxication is concerned. It should be mentioned that human placentar tissue has in our experiments given the same negative results.

SUMMARY

The liver of fetal rabbits has been tested for its glucuronide detoxication power. It appeared that the rabbit liver is able to perform glucuronide synthesis at the time of birth and that this ability is first present during the later half of the intra-uterine life.

The placenta of the same animal did not possess the power to perform glucuronide synthesis in vitro.

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FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF HELSINKI

SENSITISATION TO MORPHINE BY EXPERIMENTALLY INDUCED ALCOHOLISM IN WHITE MICE

by

ILONA VENHO, RISTO EEROLA, EINO V. VENHO, and OSMO VARTIAINEN

(Received for publication November 10, 1954)

In an earlier study on mice (1) we showed that acute alcohol-morphine intoxication is due to a potentiative type of synergism between alcohol and morphine. Since morphine is frequently administered to chronic alcoholic addicts, a study of the toxic effects of morphine in experimentally-induced alcoholism in mice seemed advisable.

Technique. — The experiments were made on white female mice on one breed weighing 25—35 g each. In order to create an alcoholic addiction the mice were induced to drink alcohol daily for three and a half months. The initial alcohol concentration was 0.5 per cent and the final 10 per cent. The alcohol was given in a weak sugar solution. This was the only liquid the mice were allowed to drink, with the exception of milk given once a week. The initial alcohol dose of 0.5 per cent was soon increased to 1 per cent and then to 2 per cent. The alcohol content of the solution was increased by 2 per cent at every ten days until, at the end of the second months, the final concentration of 10 per cent was reached. The mice received a 10 per cent solution for six weeks before the administration of morphine was begun. The food consisted of a mixture containing 1.5 kg of oats, 1.5 kg of rye flour, 300 g of casein, 150 g of butter, 150 g of yeast, 70 g of mineral

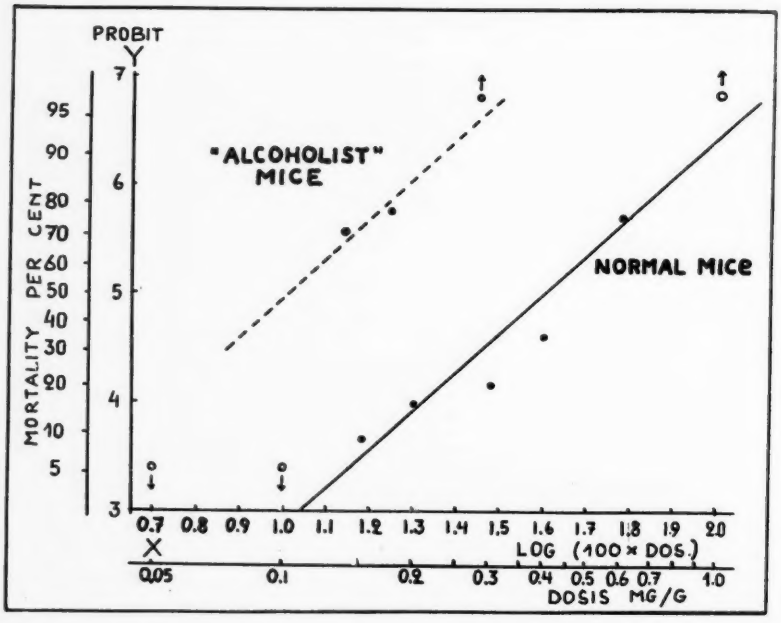

Fig. 1. — Morphine mortality illustrated by a regression line for normal mice (ca. 20 mice per dot) and the probable regression line for mice habituated to alcohol.

food mixture (food for domestic animals containing various minerals), and 5 spoonfuls of cod liver oil. The mice also received some cheese crusts every week. The control mice were kept on the same diet. The food seemed to be fully adequate. There were no signs of nutritional deficiency, and the increase in weight was at least the same as with the control mice.

In the beginning the mice proved to be unwilling to drink the alcohol solution and only took it as there was no other way to allay thirst. Later, part of the mice became so accustomed to alcohol that they drank it even when milk was available. The majority, however, preferred milk all the time. Some individuals showed symptoms of intoxication. They occasionally fell from narrow strip along which they were walking. This never happened to control mice.

Three and a half months after the mice began to receive alcohol they were given morphine subcutaneously. Before the injection

the administration of alcohol was stopped for a day to give the blood alcohol time to disappear entirely.

Using the same breed of normal mice, the mortality curve was determined for morphine and the lethal doses were calculated at 5, 10 and 50 per cent kill. (Fig. 1.) On the basis of these calculation, the alcoholic mice received LD 5, LD 10, and LD 50. The results are shown in table 1. Owing to the limited number of mice available

TABLE 1

MORPHINE MORTALITY IN MICE HABITUATED TO ALCOHOL AND MORTALITY IN NORMAL MICE CALCULATED AT A PROBABILITY OF 95 PER CENT

Dose of Morphine mg/g	Mice Habituated to Alcohol			Normal Mice
	No.	Deaths	Mortality %	Calculated Mortality %
0.14	7	5	71	5
0.18	9	7	78	10
0.40	14	14	100	50

for the experiment the series remained small; yet it showed clearly that mice habituated to alcohol were much more sensitive to morphine than normal mice. The degree of sensitisation was higher than we had expected. All mice which received a LD 50 calculated on normal mice were killed, and it is highly probable that even a considerably smaller dose, such as LD 30 for normal mice, would have been sufficient to cause 100 per cent mortality. Fig. 1 shows a regression line for normal mice and a probable regression line for mice habituated to alcohol. The lines differ greatly from one another. In spite of the limited number of the mice, the relative mortality also showed a significant statistical divergence from the mortality rate of normal individuals.

SUMMARY

The object of the investigation was to study sensitisation to morphine in white mice habituated to alcohol. The mice had been made to drink, for three months and a half, increasing amounts of alcohol, the concentration being 10 per cent for the last six weeks. The mice habituated to alcohol tolerated morphine much less than

normal mice. LD 5 calculated on normal mice produced a mortality of 71 per cent in alcoholic mice and LD 10 a mortality of 78 per cent.

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FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF HELSINKI

ACUTE ALCOHOLIC POISONING AND MORPHINE

AN EXPERIMENTAL STUDY OF THE SYNERGISM OF MORPHINE AND ETHYL ALCOHOL IN MICE

by

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(Received for publication November 10, 1954)

The Finns use alcoholic drinks relatively seldom, but when they do, they usually take strong drinks in large quantities. This accounts for the rather frequent occurrence toxic symptoms. It is a widespread habit among medical practitioners in this country to prescribe morphine for controlling states of mental unrest during inebriation, particularly during its late stages.

On the basis of clinical observations in the year 1951 one of us (O. V.) suggested to the Research Council of Alcoholic Problems [1] that a special study of the synergism of alcohol and morphine be undertaken. He had found that even therapeutic doses of morphine seemed to have an unexpectedly strong effect, and it also seemed possible that the administration of morphine had been the cause of death in some cases of alcoholism.

Møller (4) reported a number of deaths in Denmark in which the fatal issue was evidently due to a therapeutic dose of morphine or of morphine and scopolamine administered to a person under

[1] Our thanks are due to the Research Council of Alcoholic Problems for financial support.

the effect of alcohol or barbiturate and bromine. The literature does not seem to contain any mentions of the synergistic action of alcohol and morphine, while there are several reports on the synergism of alcohol and barbiturates (2, 5, 6).

The object of the experiments here reported was to study the simultaneous toxic action of alcohol and morphine.

TECHNIQUE

The mice used for the experiments were female white mice of the same breed and weighed 25—35 g. The morphine was injected subcutaneously as a 2 per cent solution and the alcohol was administered by a cannula direct into the stomach (Behrens's method) as a 20 per cent solution (1). Post-mortem examinations showed that 20 per cent alcohol did not noticeably irritate the stomach, while a 30 per cent solution produced hemorrhagic gastritis. Solutions of less than 20 per cent were found unpractical because the weakness of the solution necessitated the use of such quantities that the accuracy of dosage and the technique of administration suffered.

The lethal curves for alcohol and morphine were first determined separately, and from these we calculated a number of lethal doses withing the range of LD5—LD95. Lethal doses of morphine and alcohol calculated within these 95 per cent confidence limits where then administered to mice and the number of deaths was registered.

BASIC OBSERVATIONS AND STATISTICAL ANALYSIS[1]

The experimental data are shown in table 1. The values of the log doses x_1 and x_2 used in the calculation are given in this table.

The experiments with alcohol are illustrated in Fig. 1, in which the dots represent the probits of the death rate. The small arrows indicate doses with 0 per cent kill (later, respectively, 100 per cent kill). The illustration shows the probit regression line. A summary of the computations follows, giving all the necessary quantities

[1] The statistical calculations were carried out by Mr. Jaakko Kihlberg, M. A. Our sincere thanks are due to him for his valuable assistance.

TABLE 1

BASIC OBSERVATIONS

Alcohol Dose ml/g	Morphine Dose mg/g	x_1	x_2	Number of Animals		Mortality per cent	Empirical Probit Value
				Total	Killed		
Alcohol Experiment							
0.005	.	0.70	.	15	—	—	$-\infty$
0.006	.	0.78	.	20	—	—	$-\infty$
0.007	.	0.85	.	15	2	13	3.87
0.0075	.	0.87	.	16	5	31	4.50
0.009	.	0.95	.	20	10	50	5.00
0.01	.	1.00	.	20	17	85	6.04
Morphine Experiment							
.	0.05	.	0.70	5	—	—	$-\infty$
.	0.1	.	1.00	5	—	—	$-\infty$
.	0.15	.	1.18	23	2	9	3.66
.	0.2	.	1.30	20	3	15	3.96
.	0.3	.	1.48	5	1	20	1.46
.	0.4	.	1.60	20	7	35	4.61
.	0.6	.	1.78	20	15	75	5.67
.	0.8	.	1.90	10	10	100	∞
Combined Experiment							
0.005	0.1	0.70	1.00	20	3	15	3.96
0.0064	0.14	0.81	1.15	23	10	43	4.82
0.0074	0.14	0.87	1.15	23	12	52	5.05
0.0082	0.14	0.91	1.15	22	15	68	5.47
0.0089	0.14	0.95	1.15	15	15	100	∞
0.0064	0.29	0.81	1.46	20	15	75	5.67
0.0074	0.29	0.87	1.46	20	18	90	6.28
0.0064	0.40	0.81	1.60	15	15	100	∞

and a short variation analysis (3). On the basis of the calculated regression line some doses at certain convenient lethality levels were chosen for further experiments. These doses are shown at the end of the summary together with 95 per cent confidence belts for the expected percentage of deaths. The figures »calculated %» refer to the regression line fitted into the data.

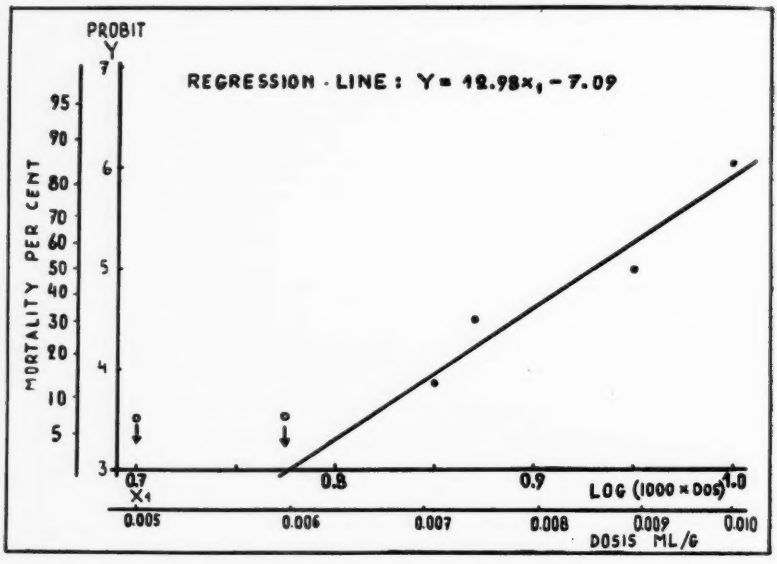

Fig. 1. — Alcohol experiment.

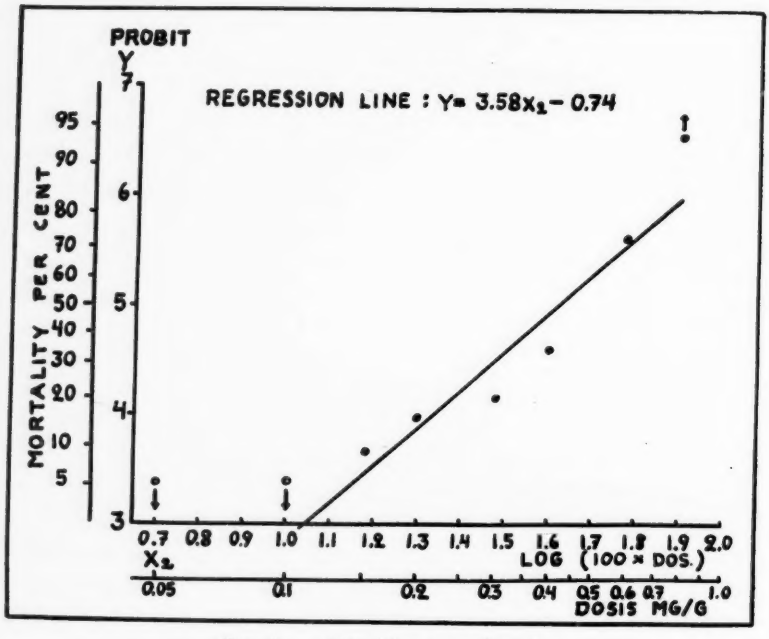

Fig. 2. — Morphine experiment.

Summary of the Results for Alcohol. — Regression equation:

$$Y = 12.98x - 7.09,$$

where

Y = probit;
$x = \log_{10}$ (dose ml/kg);
χ^2 for heterogenity = 1.93; $P > 0.1$;
$s\,(a) = 0.15$; $s\,(b) = 2.05$.

The experiment with morphine was treated along similar lines, as shown in Fig. 2 and in the summary below. Here, too, confidence belts for the expected mortality percentage were calculated.

Summary of the Results for Morphine. — Regression equation:

$$Y = 3.58x - 0.74,$$

where

Y = probit;
$x = \log_{10}$ (dose mg/100 g);
χ^2 for heterogenity = 5.00; $P > 0.1$;
$s\,(a) = 0.16$; $s\,(b) = 0.70$.

In an experiment in which alcohol and morphine are used simultaneously, one may set up a »null hypothesis» so that, for example, the effects of the two drugs are presumed to be independent and additive in the sense that LD 5 of alcohol together with LD 5 of morphine produces approximately $5 + 5 = 10$ per cent mortality. The expectations based on this hypothesis and on previous experiments are, of course, subject to random fluctuations. We therefore calculated a 95 per cent confidence belt for the expected mortality rate based on the null hypothesis. These belts are shown in Table 2,

TABLE 2

95 PER CENT CONFIDENCE BELTS FOR MORTALITY IN THE COMBINED EXPERIMENT ON THE NULL HYPOTHESIS, THAT THE EFFECT OF THE DRUGS IS PURELY ADDITIVE AND INDEPENDENT; AND THE ACTUAL MORTALITY

Morphine Dose mg/g		Alcohol Dose ml/g			
		0.0064	0.0074	0.0082	0.0089
0.14	Confidence belt	1 . . . 38	6 . . . 58	16 . . . 77	32 . . . 90
	% kill observed	44	52	68	100
0.29	Confidence belt	14 . . . 63	29 . . . 71	.	.
	% kill observed	75	90	.	.
0.40	Confidence belt	30 . . . 79	.	.	.
	% kill observed	100	.	.	.

together with the actual results from the combined experiment. As a rule, in 5 cases out of 7 our results fell outside these belts, thus showing a definite disagreement with the null hypothesis.

To make the point clearer, we calculated the expected frequencies of animals killed and alive on the null hypothesis and then compared them with the frequencies actually observed. This analysis is shown in Table 3. Owing to the small number of the

TABLE 3

TESTING THE NULL HYPOTHESIS THAT THE EFFECT OF THE DRUGS IS PURELY ADDITIVE AND INDEPENDENT

Morphin Dose mg/g	Alcohol Dose ml/g	Number of Animals					
		Expected			Observed		
		Killed	Alive	Total	Killed	Alive	Total
0.14	0.0064–0.0074	8.0	38.0	46	22	24	46
0.14	0.0082–0.0089	19.6	17.4	37	30	7	37
0.29–0.40	0.0064–0.0074	25.3	29.7	55	48	7	55

Degrees of freedom $= 5$

$\chi^2 = 79.2$; $P < 0.001$

animals it was necessary to combine some groups in order to make the χ^2-test adequate, and these combinations are shown in the table. The quantity χ^2 observed is so large as to allow rejection of the null hypothesis on a rather high level of significance. The conclusion is thus: our data are definitely and statistically significantly in disagreement with the null hypothesis specified above, so that the joint effect of alcohol and morphine does not seem to be of the simple additive type presumed in the hypothesis.

In this analysis, we assumed that the joint effect of these drugs can be described by means of a probit plane; accordingly, a probit regression analysis with two arguments has been carried out. The data from the observations have been given above; the principal results will be given below. In Fig. 3 the calculated probit plane is shown by means of four lines. The less steep lines indicate the effect of morphine at constant alcohol doses; the steeper lines indicate the effect of alcohol at constant morphine doses. Numerical

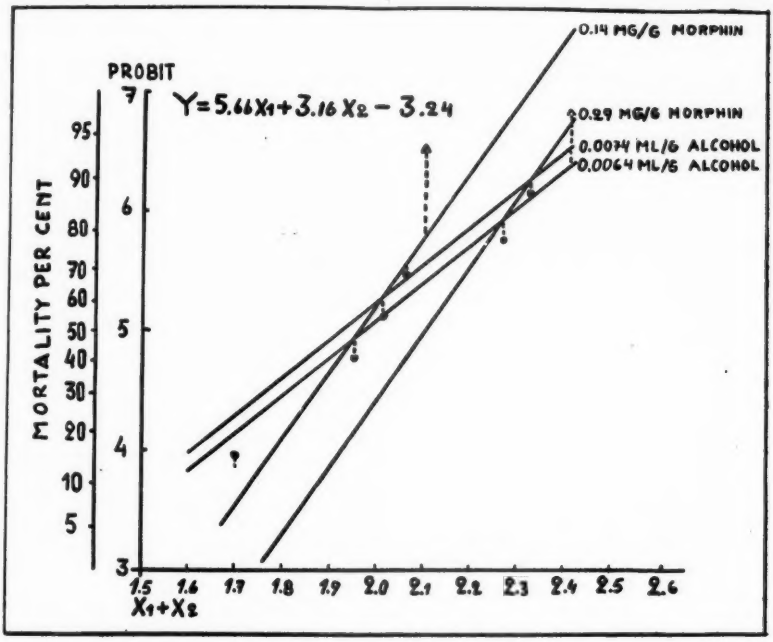

Fig. 3. — The probit plane, combined experiment.

values of the constant doses are shown in the illustration to facilitate the localisation of the plane. Note that the x-axis does not give x_1 or x_2 alone, but the sum of the two. The dotted lines from the observed frequency points to the regression lines give an idea of residual variation. A closer analysis is made in the summary of calculations. To improve the visualisation, another graph (Fig. 4) has been prepared. Here, the log dose axes x_1 and x_2 are shown together with the actual dose values. The percentage lines drawn in the x_1, x_2 field are »altitude curves» of the probit plane, showing the percentage of deaths which could be expected on the basis of the regression equation. Among these lines, dots with percentage figures attached show the agreement of the data with the fitted plane. To give an idea of how these results deviate from the null hypothesis we have drawn along the axes additional percentage scales showing the expected mortality rates at different dose levels when the drugs are used alone (these percentage scales refer to the regression lines computed from the data of the previous experi-

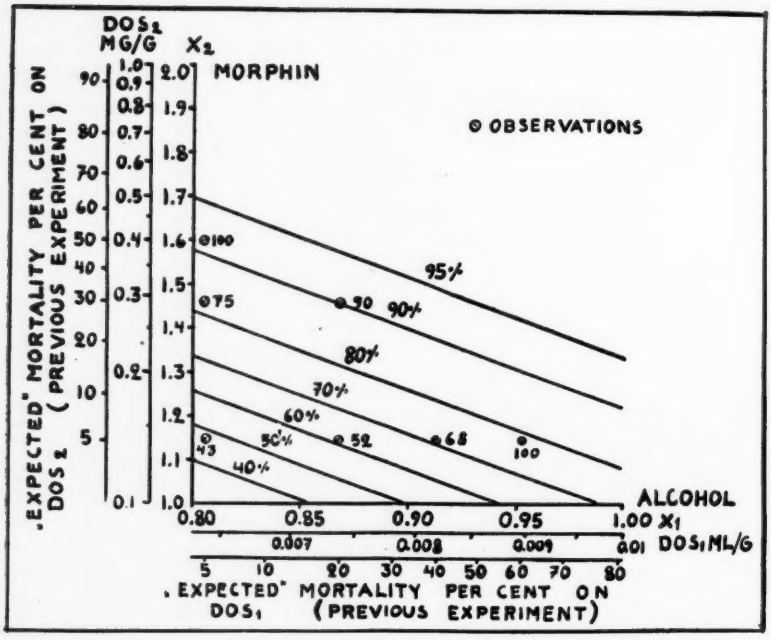

Fig. 4. — The probit plane, showing expected mortality at different dosage levels.

ments). Even if both these scales and the altitude curves of the joint probit plane are subject to random errors, the magnitude of the differences can easily be seen. The measures of variability for the two previous regression lines have already been given. As to the probit plane, we calculated the 95 per cent confidence belt for the expected percentage of deaths at one point only. Thus, 0.0070 ml/mg of alcohol together with 0.14 mg/g of morphine (corresponding roughly to LD 10 and LD 5, respectively) should, according to the probit plane, produce a mortality of 50 per cent. This figure is a random variable, and it appears that a confidence belt of 36 . . . 64 per cent would cover the true value with 95 per cent probability. In greater distances from the centre of observations the error bands rapidly grow larger and larger.

Summary of the Results in the Combined Experiment. — Regression equation:

$$Y = 5.66\, x_1 + 3.16\, x_2 - 3.24,$$

where

Y = probit;
$x_1 = \log_{10}$ (alcohol dose ml/kg);
$x_2 = \log_{10}$ (morphine dose mg/100 g);
χ^2 for heterogenity = 9.32; $P > 0.05$;
$s\,(a) = 0.11$; $s\,(b_1) = 1.46$; $s\,(b_2) = 0.63$.

SUMMARY

The object of the investigation was to study any toxic effects in white mice of simultaneously administered alcohol and morphine. The statistical analysis of the results shows clearly that the joint effect of ethyl alcohol and morphine is not the sum of the effects of these drugs when administered separately but that a potentiative type of synergism exists between the effect of ethyl alcohol and that of morphine. The potentiation was more distinct with small than with large doses. In cases in which the additive mortality would have been 10—25 per cent with small doses, it was nearly three times higher. With largest doses the mortality was approximately twice that of additive mortality. The results of the study suggested further that when the morphine dosage remains constant and alcohol is given in ascending LD's, mortality rises more rapidly than when the alcohol dose remains constant and lethal doses of morphine are increased. Statistically this is not, however, absolutely certain.

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FROM THE DEPARTMENT OF PHARMACOLOGY AND FROM THE WOMEN'S CLINIC, UNIVERSITY OF HELSINKI

EFFECTS OF PROGESTERONE, DESOXYCORTICOSTERONE AND ESTRADIOL ON THE PITUITARY AND ADRENAL GLANDS IN OOPHORECTOMIZED GUINEA PIGS

by

VEIKKO J. UUSPÄÄ and PENTTI A. JÄRVINEN

(Received for publication November 22, 1954)

It is well known that repeated doses of adrenal cortical extract or pure corticosteroids are able to inhibit the release of ACTH from the pituitary and cause a compensatory atrophy of the adrenal cortex. 11-desoxycorticosterone (DCA) is less effective in this respect than 11-oxycorticosteroids. The latter steroids also cause the thymus involution, while DCA has a very slight action on thymus (for ref. see 11, 15). Progesterone, which is chemically closely related to DCA, also possesses DCA-like biological activity to some extent, though it is more feeble than that of DCA (for ref. see 4). The effects of progesterone on pituitary and adrenal glands have, however, varied in different animals. Selye, Browne and Collip (17) noted that progesterone induced an enlargement of the pituitary in young female rats, while the weight and histological appearance of the adrenals remained normal. Brooksby (2, 3) noted equally the hypertrophy of the pituitary after the prolonged administration of progesterone in spayed rats. Clausen (5, 6, 7) discovered, on the other hand, that in adult male rats progesterone induced the atrophy of the adrenal cortex and diminution of cells of the fasciculate and reticular zones. These changes did not occur in hypophysectomized animals, so that the effect of progesterone obviously took place through the anterior pituitary gland. Also Selye and Albert (16) and Winter (19) paid attention to the involution of the adrenals caused by progesterone in male rats. According

to Kimeldorf and Soderwall (10), on the other hand, progesterone induced the enlargement of the adrenals in spayed guinea pigs.

On the contrary, it is unanimously agreed that the estrogens stimulate the anterior pituitary cells directly or undirectly, causing the increased secretion of ACTH and the enlargement of the adrenal cortex (for ref. see 4).

THE PRESENT SERIES

The guinea pigs were spayed by the dorsal route under light ether anesthesia. The first series consisted of 15 animals that were spayed five months prior to the start of the administration of the hormones. The animals were divided into three groups, each one consisting of five guinea pigs. The animals in the first group were injected subcutaneously 5 mg of progesterone (= 5.7 mg/kg) in 0.5 ml of olive oil daily for 14 days. The second group was administered 5 mg of progesterone (= 5.8 mg/kg) and, in addition, 0.25 mg of estradiol benzoate (= 0.29 mg/kg) daily and the rest of the animals were injected subcutaneously 0.5 ml of olive oil daily during equally long period. The animals were killed by bleeding after a blow on the head a few days after the last injection. The adrenal and pituitary glands and some other organs were removed, cleaned from the connective tissue and weighed. The left adrenal, pituitary and thyroid, and samples of the other organs were fixed in 10 per cent formalin solution. From the other adrenal an extract (1 : 20) was prepared by 0.1 N HCl for the adrenaline and noradrenaline determinations. These results will be discussed later in another connexion.

The results of the weight measurements of the pituitary and adrenal glands, given as the means of the groups, are summarized in Table 1. Fig. 1 demonstrates the same results in the entire series. The average weight of the pituitary glands in the progesterone group was slightly (18.3 per cent), but significantly ($t = 3.87$), lower than that of the control group. Progesterone, in dosage used, had no effect upon the weight of the adrenals in spayed guinea pigs.

The animals which were administered progesterone and estradiol simultaneously in dosage ratio 20 : 1 showed the average relative weight of pituitary glands (2.11 mg/100 g body weight) approxi-

TABLE 1

THE EFFECT OF PROGESTERONE, ESTRADIOL AND DCA ON BODY WEIGHT AND PITUITARY AND ADRENAL WEIGHT

Treatment	Number of Animals	Final Average Body Wt.	Average Percentile Change of Body Wt.	Average Weight of Pituitary		Statistical Significance	Average Weight of Adrenals		Statistical Significance
				Absol. Wt. g	Relat. Wt. mg/100 g Body Wt.		Absol. Wt. g	Relat. Wt. mg/100 g Body Wt.	
First Series									
Controls	5	737	— 0.7	15.3	2.08		441	59.8	
5 mg of progesterone daily for 14 days	5	846	— 3.4	14.4	1.70	S	511	60.4	
5 mg of progesterone and 0.25 mg estradiol benz.	4	828	— 4.4	17.3	2.11		593	72.0	
Second Series									
Controls	5	553	+11.7	12.9	2.33		310	56.1	
10 mg of progesterone daily for on an average 25 days.....	5	579	+18.2	10.3	1.80	S	367	63.4	
0.25 mg of estradiol benz. daily for 25 days	5	557	— 1.2	16.3	2.93	S	451	81.0	S
50 mg of cryst. DCA (single dose).....	5	645	+23.6	11.8	1.83	S	348	54.0	

mately similar to that of the controls (2.08 mg/100 g). The adrenals, on the other hand, were somewhat enlarged (20.4 per cent), though the change was of slight statistical significance due to the great scatter ($t = 1.72$). The fifth animal in this group died of lymphadenitis colli (septicemia ?) after the 14th injection. It had lost weight considerably (22.4 per cent) and its adrenals were greatly enlarged (the absolute weight 720 mg, the relative weight 122 mg/100 g). This case was not included in the discussion of the results.

The weather was rather cold during these experiments (in February, 1954) and the temperature tended to fall beneath 10° C in the animal quarters, remaining on two days at intervals of a week at 6° C only, for about 12 hours. Accordingly, there exists an evidence that a chronic cold stress had an influence on the results. It seemed therefore to be necessary to conduct another series of experiments on the animals that had not been under influence of the cold stress before or during the experiment. Twenty

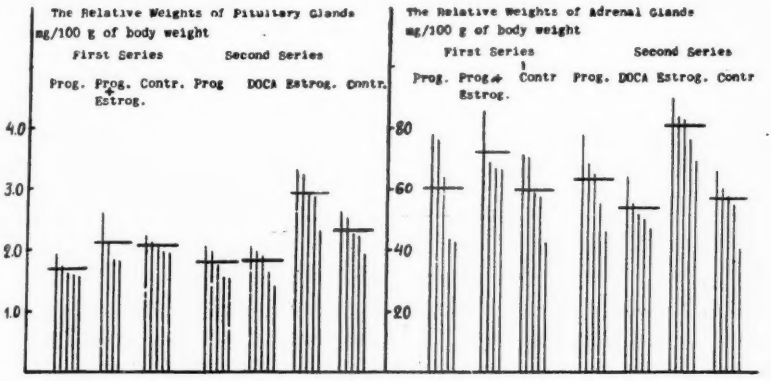

Fig. 1.

female guinea pigs were utilized in the other series, spayed in the same manner as earlier. Four groups, each of them consisting of five animals, were formed. The administration of hormones was started four weeks after the operation. The animals in the first group were injected subcutaneously 10 mg of progesterone (Lutocyclin Ciba), on an average 20.4 mg/kg, daily, and the second group was administered 0.25 mg (= 0.44 mg/kg) of estradiol benzoate for 18—30 days, until they were killed. The animals in the third group were injected subcutaneously 50 mg of crystalline DCA (Percorten cryst. Ciba) on the first day of the hormone treatment in a single dose, and the guinea pigs in the fourth group were administered 0.4 ml of olive oil. All the animals endured the operation and hormone treatment well. One guinea pig from each group, with the exception of the DCA group, was killed always on the same day and the organs were studied in the same manner as in the former series. Two animals in the DCA group were killed 28 days and the other three 38 days after the administration of the crystalline DCA. The temperature remained at 20° C during the entire experiment.

The average relative weights of the pituitary and adrenal glands in each group are shown in Table 1. Fig. 1 illustrates graphically the weights in the various cases. The pituitary glands were, on an average, of the same weight in animals treated with progesterone or crystalline DCA, being, however, slightly (22.7 and 21.5 per cent, respectively), but statistically significantly or almost sig-

TABLE 2

THE AVERAGE THICKNESS OF THE ADRENAL CORTEX AND ITS ZONES IN THE DIFFERENT GROUPS ON THE BASIS OF MICROSCOPICAL MEASUREMENTS

Treatment Group	Adrenal Cortex (mm)	Percentile Change	Glom. + Fasciculate Zones (mm)	Percentile Change	Proportion of Glom. + Fasc. Zones (%)	Reticular Zone (mm)	Percentile Change	Proportion of Reticular Zone (%)
Controls	1.03	—	0.74	—	71.8	0.29	—	28.2
Progesterone	0.93	— 9.7	0.61	—17.6	70.9	0.32	+ 10.3	29.1
Estradiol benzoate	1.68	+63.1	1.07	+44.6	63.7	0.61	+110.3	36.3
Progesterone and estradiol benzoate	1.25	+21.4	0.78	+ 5.4	62.4	0.47	+62.1	37.6
Desoxycorticosterone ..	1.11	+ 7.8	0.78	+ 5.4	70.3	0.33	+13.8	29.7

nificantly (t = 3.4 and 2.9, respectively) lighter than those of the controls. The pituitary and adrenal glands of the animals treated with estradiol were, on an average, significantly greater (25.8 and 44.4 per cent, respectively) than those of the control animals. A statistically insignificant rise occurred in the weight of the adrenals in animals treated with progesterone as compared with the control animals. The body weight of the animals treated with progesterone or DCA increased somewhat more than that of the controls, while the animals treated with estradiol gained very little weight (two guinea pigs) or lost it (three guinea pigs).

Histological Comparison. — The histological specimens were prepared from the pituitary and the left adrenal of each animal by using various staining methods (van Gieson's stain, Delafield's hematoxylin-eosin and, from the adrenal, in addition, Sudan stain). The following results concern the adrenals only. The thickness of the adrenal cortex was measured microscopically. The reticular zone was measured separately and the thickness of the glomerular and fasciculate zones together was calculated by subtracting the thickness of the reticular zone from that of the whole cortex. The means of the determination results are summarized in groups in Table 2. The values are corrected according to the average body weights of the groups. The zone limits were often rather unclear; accordingly, the results must be evaluated with a certain reservation.

In animals treated with estradiol benzoate the adrenal cortex

was, on an average, 63 per cent, the fasciculate zone 45 per cent, and the reticular zone 110 per cent thicker than in the control group. The results in Table 2 give the impression that progesterone would have had a slightly inhibitory action (—18 per cent) on the fasciculate zone, but simultaneously a very slightly stimulating or, at least, a maintenant action on the reticular zone. The animals that were administered progesterone and estradiol benzoate in the ratio of 20: 1 simultaneously had relatively somewhat thicker (21 per cent) adrenal cortex than the controls; the reticular zone being 62 per cent and the fasciculate zone only 5.4 per cent thicker than in the control glands. The comparison of these figures with the corresponding figures of animals that were administered estradiol only showed that the action of estradiol was considerably smaller in the former group. The antagonism became most evident in the fasciculate zone.

The chronic treatment with a single dose (50 mg) of crystalline DCA showed no significant effect upon the adrenal cortex.

DISCUSSION

In both the series, progesterone, used in large doses, caused the decrease in the weight of the pituitary in spayed guinea pigs. It is known that gonadectomy is followed by enlargement of the pituitary and adrenal glands [for ref. see Burrows (4)]. Selye and co-workers (17) pointed out that in intact female rats, which were injected subcutaneously 4 mg of progesterone daily for 12 days, the average weight of the pituitary was greater than that of the controls, the weight and histological appearance of the adrenals remaining unchanged. Moreover, atrophy of the ovaries and thymus occurred in the series of Selye and co-workers. In spite of the diminution of the pituitary, atrophy of the adrenals did not occur in guinea pigs treated with progesterone in the present series, unless the narrowing of the fasciculate zone, occurring in the histological appearance, would not be considered such. The result is in disagreement with Clausen's (5, 6, 7) observations on male rats, in which progesterone induced atrophy of the adrenals as a result of the inhibition of ACTH secretion. To some extent, though not statistically significantly, our results seem to suggest similar findings as obtained by Kimeldorf and Soderwall (9), who noticed an enlargement of the

adrenals in spayed guinea pigs caused by very small progesterone doses (0.05—0.2 mg daily).

If the results of the microscopical measurements of the adrenal cortex were considered to be of some importance, both the opposite concepts could be supported by them. Our results suggested, to some degree, that progesterone would inhibit the fasciculate zone, but stimulate the reticular zone; a fact that would give a rise to various speculations in this rather obscure problem (*cf.* later).

The investigations on the mutual relationships of progesterone and estrogen, when administered simultaneously, seem to suggest the antagonism between these steroids concerning their effects upon the adrenals. Progesterone as well as DCA or testosterone were able to inhibit the stimulating effect of estradiol on the adrenals, when administered in large doses (1, 13). Our preliminary experiment on the four animals is not sufficient to elucidate the problem. Greater series are needed to explain the mutual relationships of these steroids in the various zones of the adrenal cortex.

It seems likely that the inhibitory action of progesterone on pituitary is similar to that of DCA, both of them resembling each other with regard to their chemical structure. It is difficult to explain the inhibitory action of progesterone on the pituitary and, on the other hand, the non-atrophic, perhaps even slightly stimulating, action on the adrenal cortex. It is known that the chronic treatment with progesterone inhibits the formation of gonadotrophic hormones in the anterior pituitary cells. There are controversial opinions of the action of progesterone on the ACTH secretion and the adrenal cortex. In this connexion we may perhaps refer to the results obtained recently on the isolated beef adrenal. Hechter and co-workers (9), Stone and co-workers (18) and Heard and Peron (8) have pointed out that progesterone converts to 17-hydroxycorticosterone and corticosterone in an isolated perfused beef adrenal also without the addition of ACTH [for ref. see also Pincus (12)]. If progesterone is a natural metabolic precursor in the physiological synthesis of 17-hydroxycorticosterone in the adrenal cortex, it would appear possible that also *in vivo* the increase of this precursor in the blood would likely facilitate and accelerate the synthesis of Cpd F and corticosterone in the adrenal cortex to some extent. The higher concentration of these steroids in the blood would, in turn, have an inhibitory action upon the ACTH

production of the anterior pituitary lobe. Accordingly, progesterone would have an indirectly inhibitory action also on the basophil pituitary cells which are believed to produce ACTH. On the other hand, though the effect of ACTH on the adrenals would diminish, the atrophy of the adrenal cortex is not obvious, at least in all the zones, since it has sufficient precursor for the maintenance of the specific cell function, perhaps even for its activation without ACTH stimulation. It may also be possible that there exist special cells for the enzymatic processes that need ACTH stimulation. These cells would mainly be located in the outer part of the cortex, in the fasciculate zone. On the other hand, the other cortical cells would take care of the conversion of progesterone to 17-hydroxycorticosterone, which may perhaps be possible without the action of ACTH. These cells would mainly situate in the inner part of the fasciculate zone and in the reticular zone. The direction of the enzymatic process would thus correspond to the direction of the blood flow in the adrenal cortex. However, it is not as yet possible to form a clear picture of these problems on the basis of the present data. Further hypotheses are created by observations that in rats testosterone, when used in large doses, has simultaneously an inhibitory effect on the pituitary and thymus, but an increasing effect on the adrenals (14), though it cannot, according to Hechter and coworkers (8), compensate progesterone in the synthesis of Cpd F in the perfused beef adrenal.

SUMMARY

The relative weight of the pituitary in spayed guinea pigs that were treated with progesterone was, on an average, 20.5 per cent lower than that in the control group. It appeared that progesterone, when administered in doses of 20 mg/kg, had a slightly enlarging, but statistically insignificant, effect upon the adrenals.

The pituitary and adrenal glands in the animals that were treated with estradiol benzoate were, on an average, 25.8 and 44.4 per cent, respectively, heavier than those of the control animals.

The antagonistic effect of progesterone and estradiol upon the weight of the pituitary became also evident in their simultaneous administration in the ratio of 20: 1. This dosage yielded no marked antagonistic effect between them with regard to the adrenals.

The chronic treatment with a single dose (50 mg) of crystalline desoxycorticosterone showed almost significantly depressive action on the pituitary gland, but no influence upon the adrenals.

An attempt was made to interpret the effects of progesterone on the pituitary and adrenal glands on the basis of the recent literature.

ACKNOWLEDGEMENTS

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EFFECTS OF ESTRADIOL, PROGESTERONE AND DESOXYCORTICOSTERONE ON THE THYROID GLAND IN OOPHORECTOMIZED GUINEA PIGS

by

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(Received for publication November 22, 1954)

In a recent paper (18) we studied the effects of estradiol, progesterone and desoxycorticosterone on the pituitary-adrenocortical system in oophorectomized guinea pigs. Mutual relationships have been noted between this and the pituitary-thyroid system (for ref. see 11, 12, 13). It has also been noted that some catabolic hormones, such as ACTH and cortisone, have an inhibitory action on the thyroid function, the case being the opposite with some anabolic hormones, such as testosterone and DCA (for ref. see 6, 12, 13). Therefore, it seemed justified to study simultaneously and by using the same series of experimental animals also responses of the thyroid to the afore-mentioned drugs.

MATERIAL AND METHODS

Thirty-four adult female guinea pigs were used as experimental animals. The first experiment was performed on 14 animals that were oophorectomized five months earlier by a dorsal route in slight ether anesthesia. These animals were kept at 6—10°C temperature during the experiment, which lasted for 14 days. For the second experiment 20 guinea pigs, spayed four weeks earlier, were used. The temperature in their animal quarters was 20°C and they lived in similar conditions also prior to the test.

The number and the treatment of the animals with different drugs in the various groups are recorded in the accompanying Table 1, being reported in more detail in a previous paper (18). At the end of the experiment the animals were killed by bleeding after a blow on the head. The thyroid glands were removed, cleaned from adipose tissue, weighed and fixed in 10 per cent formol. The histological specimens were prepared in the usual manner by using Delafield's hematoxylin and eosin stain. The relative amounts of the epithelium, colloid, and connective tissue were estimated by using the method of Uotila and Kannas (17).

RESULTS

Table 1 shows the changes in the average body weight during the test and the average absolute and relative weights of the thyroid

TABLE 1

Treatment	Number of Animals	Final Average Body Weight g	Average Percentile Change of Body Weight	Average Weight of Thyroid Gland		Statistical Significance
				Absolute Weight g	Relative Weight mg/100 g BodyWt.	
First Series						
Controls	5	737	— 0.7	118.4	16.1	
5 mg progesterone daily for 14 days	5	846	— 3.4	128.4	15.1	
5 mg progesterone and 0.25 mg estradiol benz.	4	828	— 4.4	65.8	7.9	S (t=4.4)
Second Series						
Controls	5	553	+11.7	62.2	11.2	
10 mg progesterone daily for in average 25 days	5	579	+18.2	67.6	11.7	
0.25 mg estradiol benz. daily for 25 days	5	557	— 1.2	44.2	7.9	S (t=3.2)
50 mg cryst. DCA (single dose)	5	645	+23.6	73.2	11.3	

glands in the various groups. Progesterone and DCA had no effect upon the thyroid weight. On the other hand, estradiol benzoate produced a significant decrease in the thyroid weight in both experiments (50.9 and 29.6 per cent, respectively). This effect of estradiol

on the thyroid was not inhibited by progesterone in dosage ratio 1:20.

The proportions of the thyroid epithelium, follicle colloid and stroma are summarized in Table 2. Progesterone or DCA had no significant effect on the epithelium percentage. Estradiol induced a decrease of 27.7 per cent in the epithelium proportion ($t = 3.5$); epithelial cells were flattened and follicles enlarged. Also in the other animals of the second series, the thyroid epithelium was rather low cuboidal and the follicles were abundant with colloid.

TABLE 2

Animal Group	Epithelium (%)	Colloid (%)	Stroma (%)
First Series			
Controls	54.2	39.4	6.4
Progesterone group	59.5	34.6	5.9
Estradiol + progesterone	53.2	40.2	6.6
Second Series			
Controls	42.9	51.7	5.4
Progesterone group	48.6	44.4	7.0
Estradiol group	31.0 ($t = 3.5$)	63.8	5.2
DCA group	40.7	51.1	8.2

DISCUSSION

The weight of the guinea pigs that were treated with estradiol decreased slightly in two and increased in three animals, though much less than in the controls. Since the thyroid weight and epithelium percentage decreased in animals treated with estradiol as compared with the controls, it cannot be assumed that estradiol had influenced the body weight through the thyroid. As estradiol strongly stimulates the pituitary and adrenal cortex (18), it is very likely that it was the increased production of catabolic adrenocortical hormones which inhibited the normal rise in the body weight.

In rats the castration induces diminution of the thyroid gland (5) and enlargement of the follicles, flattening of the follicle epithelium and increase in the amount of colloid (2). In castrated guinea pigs, on the other hand, an increased production of thyrotrophin has been noted from the pituitary and proliferative activity in the

thyroid (4). On the basis of the present material, the effect of castration on the thyroid gland cannot be established with certainty. The controls in the second series had, however, a rather low epithelium percentage (42.9 per cent) as compared, *e.g.*, with the controls in Kuusisto's series (6), which were not castrated and which were estimated by the same method (62.3 per cent).

A remarkable difference can be noted in the relative thyroid weights between the control groups of the first and second series. It is probably a manifestation of the specific response of the thyroid to cold. The epithelium percentage, too, was significantly greater ($t = 5.7$) in the control animals of the first than of the second series. Likewise, the epithelium percentage was significantly greater ($t = 3.8$) in the guinea pigs treated with progesterone and exposed to cold than in the animals which were kept at the room temperature.

The chronic treatment with large doses of estradiol benzoate produced a significant decrease in the thyroid weight and epithelium percentage in the animals which were kept at 20° C. Estradiol, when administered with progesterone in the dose ratio 1: 20, produced a 50 per cent decrease in the thyroid weight in animals exposed to cold, but no decline in the epithelium percentage. It is probable that it is the effect of cold, rather than progesterone, which antagonized the action of estradiol upon the epithelium, since progesterone alone had a very slight, but statistically insignificant, stimulating effect on the glandular epithelium. Money and co-workers (8, 9) have also paid attention to the fact that progesterone, opposite to estrogens, increases the radioactive iodine uptake of the rat thyroid.

The action mechanism of the antithyroid function of estrogen can, for the present, be explained by various hypotheses. Firstly, estrogens could suppress the thyroid function through the pituitary-adrenocortical system. ACTH, cortisone, and some stressor agents depress markedly the thyroid function probably by decreasing the secretion of thyrotrophic hormone from the pituitary. Also in adrenalectomized rats ACTH has been capable of decreasing the fixation of radio-iodine in the thyroid, thus inhibiting directly the secretion of thyrotrophic hormone. Estrogens, in turn, have a strongly stimulating action upon the ACTH secretion and the adrenal cortex. It is not known in which way ACTH produces the

decrease in the TTH secretion, though there exist various interpretations. Cortical steroids could influence sparingly thyroxin metabolism so that the amount of circulating thyroxin would increase and cause an inhibition in the production of thyrotrophic hormone. A so-called »shift in anterior-pituitary hormone secretion» (12, 13) during estrogen stress might also be in question. Because of the highly increased discharge of ACTH during stress, the production of other pituitary hormones, such as somatotrophin, gonadotrophins, and thyrotrophin, decreases (12).

That the estrogens would cause a formation of thyroid inhibiting substance in the pituitary (*cf.* 10), might be another explanation for the antithyroid effect of estrogen.

There is also a third possibility, *viz.*, that estrogens would influence, at least partly, the thyroid function through the hypothalamo-pituitary axis. Harris (3) has presented a hypothesis that variations in the blood level of estrogen exert their effects on the secretion of gonadotrophic hormone by an action on the hypothalamus rather than by an action directly on anterior pituitary cells. Uotila (16) has pointed out that the basic thyrotrophic secretory rhythm of pituitary is controlled humorally by variations in the blood thyroxin level without the mediation of the hypothalamic-hypophyseal pathway. During cold stress, however, this basic secretory rhythm may be modified by stimuli originating from hypothalamus through the pituitary stalk. Uotila (14, 15) has noted, further, that the transection of pituitary stalk prevents an increase in thyroid cell height, but not in the glandular weight in rats exposed to cold. According to Greer (1), the thyrotrophic hormone consists of two components, the one of which, »the metabolic factor,» is independent of the hypothalamus and enables the thyroid to bind iodine, while the other component, »the growth factor,» regulates thyroid cell height and thyroid size and seems to depend upon the hypothalamus.

Our results of the inhibitory action of estrogen stress on the thyroid weight and epithelium might perhaps be explained most plainly by shift in anterior pituitary hormone production. In accordance with the results obtained by others, we have noted earlier (18) in the same animals that estradiol caused a strong enlargement of pituitary and adrenal cortex. It is obvious that estradiol stimulates strongly the ACTH production in the anterior pituitary.

The simultaneous diminution of the thyroid well corresponds to Selye's theory that the shift in anterior pituitary secretion takes place during stress. The thyroid hypofunction simultaneously with the hyperfunction of the adrenal cortex would seem suitable also as to acquirement of the resistance, as it has been noted that thyroidectomy increases the resistance of laboratory animals, while the administration of thyroxin renders them sensitive again (7). On the contrary, it is not easy to explain, without subsidiary hypothesis, the modifying effect of the additional stressor agent, cold, on the antithyroid action of estradiol. In this respect, our experiment was preliminary; accordingly, the interpretation of our results has to be considered a working hypothesis. The results would appear to be explained by Greer's (1) dual theory on the thyrotrophic hormone. We should thus assume that estrogens influence the anterior pituitary through the hypothalamus and that massive doses might perhaps block hypothalamic centers controlling the thyrotrophic »growth factor.» The basic production of the thyrotrophic »metabolic factor» was maintained by increased consumption of circulating thyroid hormone caused by cold. That estrogens could simultaneously stimulate other hypothalamic centers and depress the others, might perhaps be an expression of the hypothetical shift in hypothalamic hormonal stimuli release into hypophyseal portal vessels. The better understanding of the action mechanism of estrogen is possible only on the basis of further experimental data obtained by pituitary stalk section.

SUMMARY

Estradiol benzoate, when injected subcutaneously in average doses of 0.29 mg/kg or 0.44 mg/kg for 14—30 days, produced a remarkable decrease, on an average 40 per cent, in the thyroid weight and a decline of 28 per cent in the epithelium percentage in oophorectomized adult guinea pigs. The body weight of the guinea pigs treated with estradiol remained lower than that of the others. This inhibitory effect of estradiol on the body weight could not take place through the thyroid, but through the adrenals.

Progesterone, when administered in average doses of 5.7 mg/kg or 20.4 mg/kg, caused no changes in the thyroid weight in spayed guinea pigs. A slight, but hardly significant, rise in the epithelium

percentage could be noted in the progesterone group of the second experiment. Progesterone did not prevent the decrease in the thyroid weight caused by estradiol.

The chronic treatment with a single dose (50 mg) of crystalline desoxycorticosterone for 28—38 days showed no effect upon the size and histological appearance of the thyroid.

Exposure to slight cold (together with progesterone) did not prevent the decrease in the thyroid weight caused by estradiol, while it inhibited the inactivation of the epithelium as estimated on the basis of the histological appearance and epithelium percentage. This is interpreted to suggest eventually the dual control of the thyroid function.

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FLUORESCING ISLETS, ADRENALINE AND NORADRENALINE IN THE ADRENAL MEDULLA OF SOME COMMON LABORATORY ANIMALS[1]

by

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(Received for publication December 14, 1954)

Every now and then claims have been presented that there are two or more different kinds of secretory cells in the adrenal medulla (3, 4, 6, 23, 26, 27). However, these reports have not conclusively altered the general opinion that differences in the stainability or in the histochemical properties of the adreno-medullary cells are due to differences in the secretory state of otherwise similar cells, a conclusion arrived at in recent careful studies by Bennett (2) and Hillarp (21). In 1951, Bänder (1) described a staining technique by means of which he claimed to be able to differentiate between the adrenaline-containing and the noradrenaline-containing cells of the adrenal medulla. The present writer (unpublished) has not been able to confirm Bänder's histological observations; the results of Bänder's adrenaline and noradrenaline determinations also diverge from those obtained by most other workers.

As the above-mentioned writers used either complicated staining techniques difficult to reproduce or histochemical methods giving

[1] A part of this study was done during the academic year 1952—1953, which the writer spent in the Department of Pharmacology, University of Edinburgh. Many thanks are due to Professor J. H. Gaddum and to Dr. Marthe Vogt for their willing guidance and for the laboratory facilities.

[2] With technical assistance of Mrs. L. Räisänen and Mr. E. Siltanen. This study has been supported by grants from the Valtion Luonnontieteellinen Toimikunta and the Sigrid Jusélius Foundation.

positive reactions with both adrenaline and noradrenaline, it may not be surprising that they failed to demonstrate crucially two qualitatively different types of cells. In 1951, the present writer (7, 8) observed that some histochemical reactions clearly discriminated between two different kinds of secretory cells in rat's adrenal medulla. Such differences were equally well demonstrable in resting adrenals, whose medullary cells all gave an intense chromaffin reaction and whose contents of adrenaline and noradrenaline were normal, and in stimulated adrenals, in which a loss of adrenaline was experimentally induced (7, 8, 9, 10, 16). One of the histochemical methods used, i.e. fluorescence microscopy of formalin-fixed sections, has been shown to demonstrate two kinds of medullary cells also in the adrenals of the cat and the hamster (13, 14). Furthermore, both direct and indirect evidence is available suggesting that adreno-medullary cells fluorescing after treatment with formalin contain much noradrenaline and little or no adrenaline and that the other — non-fluorescing — medullary cells contain predominantly adrenaline (13, 14, 15, 16). Recent independent observations by Hillarp and Hökfelt (22) with a histochemical technique which according to these investigators selectively demonstrates noradrenaline fit well to the present writer's results.

In the present study the histochemical fluorescence technique has been applied conjointly with adrenaline and noradrenaline determinations on adrenals of some common laboratory animals. The results are in agreement with the view that the fluorescing medullary cells are the main site of storage of adrenal noradrenaline.

A brief preliminary communication on this subject has been published in Nature (10). Since then, more material has been collected. Therefore, the data presented in the preliminary note and in this paper differ somewhat from each other.

MATERIAL AND METHODS

The series comprised 86 animals: 10 cats, 9 dogs, 10 guinea pigs, 16 hamsters, 20 mice, 6 rabbits and 15 rats. The hamsters, mice and rats were killed by cutting the neck with sharp scissors without any previous treatment. Other animals were bled through the neck after a blow on the head (guinea pigs and rabbits) or under a short anaesthesia (cats and dogs). Animals of both sexes

amongst all these species have been examined but in the respects concerned in this study no sexual differences were observed.

The adrenals were freshly frozen on the tissue holder of a freezing microtome. Sections were cut at 50 μ with a cooled knife, a part of the sections being fixed in formalin for histochemical study, another part being freeze-dried for microdissection (see 11 and 12). Sections studied for fluorescence and with the earlier described silver method (9) were fixed for 2—8 hours in formalin. Sections fixed only for ca. 3 min. and washed in three rinses of distilled water were used for the histochemical demonstration of acid phosphatase, using a technique described in an earlier paper (9). Some formalin-fixed or freeze-dried sections of the rat adrenal were also studied for acid phosphatase with azo-coupling techniques (29).

Determinations of adrenaline and noradrenaline concentrations were made after chromatographic separation, with the aid of a fluorimetric method (12). In mice, these determinations were done using whole freeze-dried sections. In all other species the medullary area of each section was first dissected out and weighed, so that the catechol amines could be expressed in terms of per cent of dry medullary weight. A part of the adrenal sections from cats and hamsters were used for studying the correlation between noradrenaline and islet contents within each section (13, 14). As small pieces of either islet-rich or islet-poor areas were selected in these studies, sometimes without analyzing the whole medullary part of the section, some of these data were not suitable for the present purpose, the analyzed pieces being not representative samples of the whole medulla. All data used in the present study were obtained from several sections of each gland, the whole medullary area of each section being used. Even so, the estimates of catechol concentrations and of islet content obtained from some mid-sections of each gland are probably not representative of the whole medulla, particularly as the distributions of both the fluorescing islets and the catechols are not always even in the adrenal medulla. This is fortunately not serious from the point of view of the present problem, as the catechols and the islet content were estimated from neighbouring sections of each gland.

The relative amount of fluorescing tissue was assessed subjectively. Such kind of estimation is of course subject to considerable errors, particularly as the variations in the distribution and in

the size of the fluorescing islets tend to distort the subjective estimates. With experience in planimetric measurements of islets of varying size it is nevertheless possible to obtain reproducible area estimates in spite of variations of the mentioned kind. Thus, it will be possible after some training to state, e.g., that the area covered by the islets is with certainty more than one tenth and less than a quarter of the whole area of the medulla, or that the true value is between one third and three quarters. The subjective method, saving the enormous amount of work which had been necessary if planimetric measurements had been done on fluorescence photomicrographs, was — in spite of it's low accuracy — good enough to supply the information required in the present study.

RESULTS

Acid Phosphatase. — In normal rats, the fluorescing medullary islets are acid phosphatase negative, the rest of the medulla being strongly positive (7—9). This finding was now confirmed also with the azo-coupling techniques, in both formalin-fixed and in freeze-dried sections. In the cat, the dog, the guinea pig, the mouse and the rabbit, no inhomogeneity was seen in the medullary reaction. Adrenals of the hamster were not examined with this method.

Ammoniacal Silver Nitrate. — The fluorescing islets of the rat have been seen to darken selectively when treated with ammoniacal silver nitrate (9). Efforts to reproduce these observations with other species did not succeed. Indeed, it proved difficult to obtain the same reaction even in the adrenals of the rat, although the method worked earlier without particular difficulties. Brands of silver nitrate and/or ammonium hydroxide different from those used in the earlier work may be responsible.

Fluorescent Islets. — Fluorescence photomicrographs of adrenals from some species have been published in earlier papers (rat: 9, 15, 16; cat: 14; hamster: 13). Figg. 1 and 2 illustrate dog adrenals. The fluorescing medullary cells can be differentiated from groups of fluorescing cortical cells sometimes present in the medulla: in sections not previously exposed to ultraviolet light the cortical cells fluoresce either bright yellow or brick red while the medullary islets show a bright green fluorescence; in sections examined for

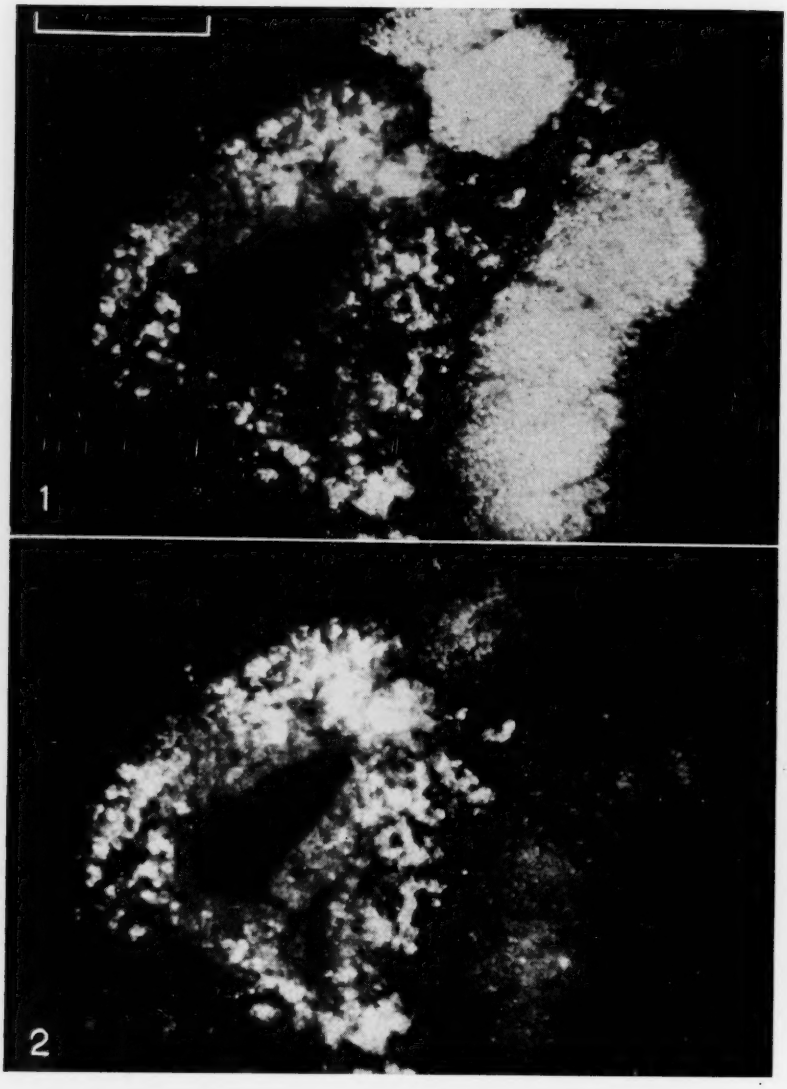

Fig. 1. — Fluorescence photomicrograph of a formalin-fixed section from the adrenal gland of a normal dog. Fluorescing materials are seen both in the cortex and in the medulla. Length of the white scale mark 300 μ.

Fig. 2. — The same section photographed again. The cortical fluorescence has largely disappeared during the exposure of Fig. 1.

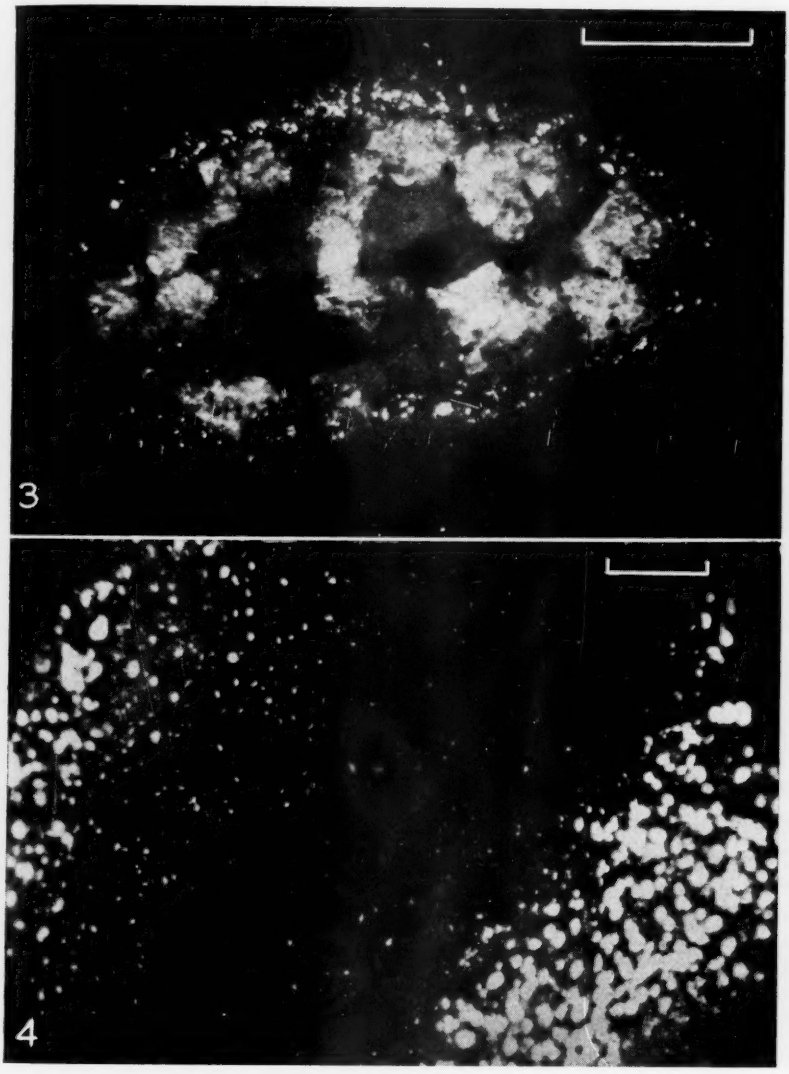

Fig. 3. — Fluorescence photomicrograph of a formalin-fixed section from the adrenal gland of a normal mouse. The cortico-medullary junction is clearly outlined by strongly fluorescing lipid-rich cells. Medullary islets are clearly visible. Empty medullary blood vessels are dark.

Fig. 4. — Similarly taken picture of the adrenal of a rabbit. The medulla, free of fluorescing materials except for some lipid-rich cells, is in the centre. The length of the scale mark in both pictures 300 μ.

some time, the ultraviolet-sensitive yellow cortical fluorescence is no more visible but the green fluorescence of the medullary islets is unaltered. Medullary islets of the mouse are illustrated in Fig. 3. A section from the adrenal of a rabbit is shown in Fig. 4.

The fluorescing islets in the species examined are briefly characterized in table 1. The estimation of the proportion of the medulla

TABLE 1

FLUORESCING ISLETS IN THE ADRENAL MEDULLA

Species (Number of Animals)	Size	Distribution of Islets	Proportion of Islets of Whole Medulla (Range)
Cat (10)	small to large	uneven	1/5—3/4
Hamster (16) ..	medium	in the periphery	1/4—1/2
Mouse (20) ..	medium	even	1/5—1/2
Dog (9)	small to large	uneven	1/4—1/2
Rat (15)	small	even	1/10—1/4
Guinea pig (10)	(small)	?	0—1/20 (?)
Rabbit (6)	—	—	0

covered by fluorescing islets was particularly difficult in the cat and in the dog because of the uneven distribution and the big variations in the size of the islets. In the guinea pig, no clear-cut islets were observed in the medulla but there were small areas fluorescing somewhat more strongly than the rest of the medulla. As the fluorescence of these areas was definitely weaker than that in the fluorescing islets of other species, it is questionable whether they are identical with the latter. In the rabbits examined, no fluorescing islets or anything resembling them were seen in the adrenal medulla, disregarding islets of cortical tissue.

Adrenaline and Noradrenaline. — Table 2 shows the concentration of both catechols as per cent of dry medullary weight and the proportion of noradrenaline as per cent of the total amount of both catechols. The over-all concentration of catechols is c. 5 per cent in the medulla of all species studied but there are marked species differences in the relative amount of noradrenaline. The figures of relative noradrenaline content in the medulla of the guinea pig and the rabbit are based on determinations on very few animals. As they do not deviate essentially from the figures re-

TABLE 2

MEDULLARY CATECHOL AMINES

Species (Number of Animals)	Adrenaline + Noradrenaline (% of Dry Weight of Medulla)		Noradrenaline (% of Both Catechols)	
	Mean	Standard Deviation	Mean	Standard Deviation
Cat (9)	5.7	0.9	40.1	11.8
Hamster (11) ..	4.2	0.7	36.5	4.8
Mouse (9)	? [1]	—	30.4	6.7
Dog (9)	5.0	0.9	29.1	4.2
Rat (15)	4.9	0.9	16.0	2.8
Guinea pig (5)	3.6	0.8	5.2	1.1
Rabbit (2)	? [1]	—	2.5	—

[1] Cortical tissue present in the samples analyzed.

ported by earlier investigators (17, 19, 20, 30—33), and as sprayed chromatograms repeatedly proved the presence of small amounts of noradrenaline in the medulla of these species, the quantitative data may be sufficiently reliable for the present purpose. The corresponding figures for the other species are also in fair agreement with figures reported earlier (5, 9, 15—20, 22, 24, 25, 28, 30—34).

Noradrenaline and Fluorescent Islets. — The percentage of fluorescing medullary tissue is plotted in Fig. 5 against the percentage of noradrenaline. It must be emphasized that the limits of islet percentage (already given in table 1) are wide enough to enclose with certainty the actual range in animals examined. The horizontal length of each rectangle, on the other hand, has been obtained by multiplying the standard deviation by the value of Student's t at the level $P = 0.05$ corresponding to the number of degrees of freedom in each group; thus, the true mean noradrenaline percentage is enclosed by each rectangel with 95 per cent probability.

In spite of the fairly large variations in both dimensions, examination of the diagram suggests a fairly good correlation between the noradrenaline content and the islet content. The significance of this correlation was tested by two methods. First, the correlation between the average noradrenaline percentage in each group and the mid-value of the group limits set for the islet content was cal-

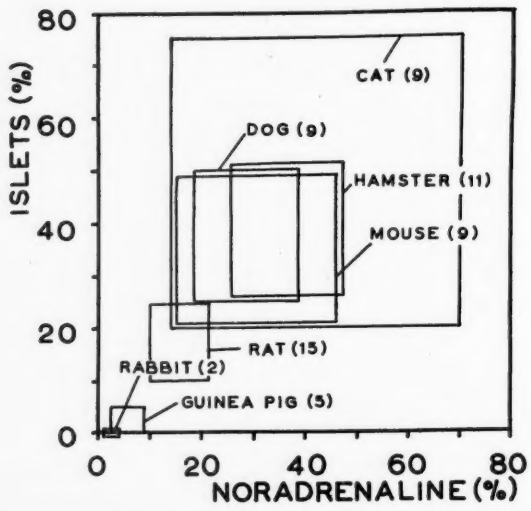

Fig. 5. — Correlation of noradrenaline content (% of both medullary catechol amines) and islet content (% of whole medullary size) in different species. For significance of the rectangles, see text. The numbers in the brackets refer to the number of animals in which catechol determinations were made.

pulated. Thus, a correlation coefficient of 0.99 was obtained, the probability that such a high correlation is due to mere chance being far less than 0.000 01.

This way of calculating the correlation is, however, subject to criticism because the mean islet content need not be exactly in the middle of the extreme limits, assessed collectively for each group. Therefore, the interdependence of noradrenaline and islet percentages was tested by the rank correlation method. The animals were first arranged into the order of decreasing noradrenaline percentage according to the means of each species. Then the animals were arranged according to the islet content, and — to be on the safe side — this was done so that the order of the hamster, the mouse and the dog was set opposite to that according to the noradrenaline percentage, a precaution rendered desirable by the fact that the noradrenaline and islet contents of these species showed considerable overlapping. Further, the order of the guinea pig and the rabbit was reversed for the same reason. The following data were thus obtained.

According to noradrenaline percentage:

1) cat, 2) hamster, 3) mouse, 4) dog, 5) rat, 6) guinea pig, 7) rabbit.

According to islet percentage:

1) cat, 2) dog, 3) mouse, 4) hamster, 5) rat, 6) rabbit, 7) guinea pig.

Comparison of the ranks:

	1	2	3	4	5	6	7
	1	4	3	2	5	7	6
Difference	0	-2	0	2	0	-1	1

Sum of squares of differences $= 4 + 4 + 1 + 1 = 10$

Rank correlation $r_s = 1 - \frac{6 \times 10}{7(49 - 1)} = 0.821$

This correlation, although it is calculated according to the most pessimistic alternative, is significant at the 5 per cent level.

DISCUSSION

The observations described support the hypothesis that the fluorescing islets are composed of adreno-medullary cells containing predominantly noradrenaline. A positive correlation between the relative islet content and the relative noradrenaline content does, of course, not provide crucial evidence. However, there are hardly reasons to believe that species such as the dog and the mouse would in this respect differ essentially from the cat, the hamster and the rat, in whose adrenals the predominant localization of noradrenaline in the islet cells has been more directly proved (13—16, see also 22). Therefore it may be justified to regard the hypothesis correct until observations speaking against it have been presented.

The non-fluorescing medullary cells of the cat and the hamster have been seen to contain mostly the methylated variety of the adreno-medullary catechol amine, i.e. adrenaline, but besides it also small amounts of noradrenaline (13, 14). In the present study, the relative noradrenaline content in the adrenal medulla of the guinea pig was observed to be ca. 5 per cent although the presence of fluorescing islets was doubtful. Noradrenaline was clearly demonstrated also in the adrenal medulla of the rabbit, in which no islets were seen. These findings fit well together and show that noradrenaline is present in cells apparently specialized in production of adrenaline, the former substance being a praecursor of the latter.

On the other hand, it is as yet not clear whether the fluorescing cells contain exclusively noradrenaline of the two medullary catechol amines or also some adrenaline. This question can be answered with certainly first when pieces of the adrenal medulla have been analyzed containing only the fluorescing variety of the medullary cells. For such a study, foetal adrenals may offer better possibilities (cf. 31, 32). The regression demonstrated in the present study between the noradrenaline percentage and the islet percentage was apparently linear, and the regression coefficient was seemingly near 1, which would suggest that the islet cells contain but little adrenaline. Owing to wide variations within the species the evidence is however insufficient in this respect. That the adrenaline content of the islet cells is at least very small in comparison to the noradrenaline content in the same cells, has been more directly demonstrated in pieces of cat's adrenal medulla with some 70 per cent of both fluorescing tissue and noradrenaline (14).

In spite of the fact the fluorescing cells apparently contain some noradrenaline and the other medullary cells possibly some adrenaline, the evidence now available leaves little doubt that two distinctly different types of endocrine cells are present in the adrenal medulla of those mammals secreting noradrenaline besides adrenaline, an idea already put forward in the papers in which the medullary islets were first described (7—9). It is interesting that Hillarp and Hökfelt (22) have through other ways arrived at fairly similar conclusions. It would be of importance to compare their histochemical reaction for the demonstration of noradrenaline with the fluorescence methods used by the present writer.

SUMMARY

Cell groups fluorescing brilliantly in ultraviolet light after fixation in formalin have been demonstrated in the adrenal medulla of the cat, the hamster, the mouse, the dog and the rat. The presence of such fluorescing islets was questionable in the adrenal medulla of the guinea pig, and no islets were detected in the adrenal medulla of the rabbit. A statistically significant between-species correlation was observed between the percentage of noradrenaline of both medullary catechol amines and the percentage of fluorescing tissue of the whole medulla. The data, together with earlier

observations, are taken to indicate that the fluorescing medullary cells contain much noradrenaline and little or no adrenaline and that the non-fluorescing cells contain much adrenaline and little noradrenaline.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY
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SEROLOGICAL REACTIONS IN RHEUMATOID ARTHRITIS

THE RELATION OF THE AGGLUTINATION ACTIVATING FACTOR (AAF) TO COMPLEMENT

by

ODD WAGER [1]

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Many rheumatoid sera display an extraordinary capacity for strongly agglutinating red cells sensitized with a subagglutinating dose of their specific antibody (7, 8, 9, *et al.*). The factor responsible for this phenomenon is relatively thermostable and belongs to the globulin fraction (7, 8, 9). There is some evidence pointing toward its association with β-globulin (10).

Some years ago, we studied the occurrence and mode of action of this factor (9). It was suggested that it might act as a kind of »agglutination complement», activating the agglutination of the red cells by their specific antibodies. The factor was called »agglutination activating factor» (AAF).

Recently, data have been presented by Gorrill and Hobson (3) suggesting a close association of AAF with the thermostable fourth component of the complement (C′4). The purpose of the present work has been to investigate whether or not confirmatory data supporting the suggested relationship between AAF and C′4 could be obtained. Separation of the components of complement by the CO_2 dilution method as well as selective inactivation of the components have been carried out, with subsequent estimation of the AAF activities of the preparations.

[1] Aided by a grant from the Sigrid Jusélius Foundation.

MATERIAL AND METHODS

Sera. — Sera from individual patients with typical rheumatoid arthritis as well as pooled rheumatoid sera exhibiting AAF activity were used for the experiments. When sera exhibiting full complement lytic activity were desired, blood samples were taken on the preceding day, and the sera stored in the icebox overnight. If not otherwise stated, inactivation of the sera was carried out by incubation at 56° C in the water-bath for 30 min.

Estimation of AAF Activity. — If not otherwise stated, the technique described elsewhere (9) was followed. According to this method, sheep cells sensitized with 0.5 MAD (minimum agglutinating dose) of specific rabbit antibody are used for the test.

Estimation of Complement Lytic Activity. — Rabbit amboceptor was standardized to contain 2.5 hemolytic units in 0.25 ml. Unit amboceptor was taken as the smallest amount necessary for complete hemolysis of 0.25 ml of a 5 per cent sheep cell suspension in the presence of 0.25 ml of fresh guinea pig complement (dilution 1 : 10). The total volume of the reactants was brought up to 1.25 ml with saline. For estimation of complement activity, 0.25 ml of the given specimen was mixed with 0.5 ml of a 2.5 per cent sheep cell suspension sensitized with 2.5 units of the amboceptor. The total volume was brought up to 1.25 ml with saline. The mixtures were briefly agitated, and the results were read after incubation for 30 min. at 37° C in the water-bath.

Inactivation of C′4 by Ammonia. — The method of Gordon *et al.* (2), with the modifications suggested by Gorrill and Hobson, was followed.

Inactivation of C′4 by Ether. — The method of Osborn (5) was followed.

Inactivation of C′3 by Zymosan. — The method of Ecker *et al.* (1) was followed. Our thanks are due to Dr. P. Grönroos, who kindly supplied the zymosan. It was prepared by him according to the method described by Pillemer and Ecker (6).

Fractionation of Complement. — The CO_2 dilution method of Liefmann (4) was followed. This gives a precipitate containing all the heat-labile first component (C′1) and the bulk of the heat-stable third component (C′3). The supernatant fluid contains all the heat-labile second component (C′2), all the heat-stable fourth component (C′4) and a trace of C′3).

EXPERIMENTS AND RESULTS

Effect on AAF Activity of Ammonia Treatment. — Ammonia selectively inactivates the fourth component of complement (C′4). In preliminary experiments, it was shown that incubation with ammonia at 37° C for 90 min. destroyed the complement lytic activity of fresh guinea pig and normal human complement. Incubation at 37° C in the water-bath without the presence of ammonia did not affect the complement lytic activity of these sera. For

TABLE 1

EFFECT OF AMMONIA ON AAF AND COMPLEMENT ACTIVITY

Rheumatoid Serum	Treatment Subjected to	Aggl. Titer for		Differential Titer	Complement Activity
		Normal Cells	Sensit. Cells		
«Va» (individual)	Incubated at 56° C for 30 min.	8	2048	256	None
»	Incubated at 37° C for 90 min. with ammonia	4	1024	256	»
»	Incubated at 37° C for 90 min. with saline				Total hemolysis
»H—K» (pool)	Incubated at 56° C for 30 min.	8	1024	128	None
»	Incubated at 37° C for 90 min. with ammonia	< 4	512	> 128	»
»	—»—	4	512	128	»
»	Incubated at 37° C for 90 min. with saline				Total hemolysis
«R—1» (pool	Incubated at 56° C for 30 min.	32	8192	256	None
»	Incubated at 56° C for 30 min, then				
»	incubated at 37° C for 90 min. with ammonia	64	8192	128	»

Complement activity was estimated as degree of hemolysis caused by the serum to be tested, previously diluted to 1 : 10.

complement activity tests, portions of the sera previously diluted to 1 : 5 and 1 : 10 were used.

Next, the effect of ammonia on AAF and complement lytic activity of rheumatoid sera was investigated. A constant volume technique was followed throughout the studies, and the necessary adjustments of volumes were carried out with saline solution.

The results are seen in Table 1. From the data presented in this

table, it can be concluded that ammonia treatment of rheumatoid sera had no effect on their AAF activity, although the complement lytic activity of the two fresh sera tested (»Va» and »H-K») was destroyed by ammonia.

According to Osborn (5), a selective inactivation of C′ 4 can also be achieved by ether treatment. One of the above sera (»Va») was subjected to treatment by ether. The complement lytic activity, however, was only moderately weakened. The AAF activity was not affected.

Fractionation of Rheumatoid Sera by CO_2 Dilution Method. — Dilution of complement serum with water and subsequent saturation with CO_2 result in formation of a precipitate. This precipitate, when redissolved in saline, contains all the C′1 and most of the C′ 3, whereas the supernatant fluid contains all the C′2 and C′4 and a little of C′3.

Three rheumatoid sera were subjected to fractionation experiments, with subsequent estimation of the AAF activities of the different preparations. According to the method of Liefmann (4), serum is diluted to 1 : 10 with water before saturation with CO_2 is started. In our hands, this method gave fractions that, when recombined, exhibited only a slight complement lytic activity. Therefore, a simultaneous fractionation (serum »Va»), using a presaturation dilution of 1 : 5, was carried out. The results in both series were similar, except that with the use of a dilution of 1 : 5 the recombined fractions exhibited a good complement lytic activity.

The results of the fractionation experiments are shown in Table 2. From this table it can be seen that by fractionation according to the CO_2 dilution method rheumatoid serum could be divided into two fractions, both devoid of complement lytic activity. On recombining the two fractions, the complement activity could be restored. Both fractions exhibited AAF activity, the precipitate fraction possibly somewhat more than the supernatant fraction. Treatment of the fractions with ammonia did not alter the AAF activity in any consistent manner.

Effect of Zymosan on AAF Activity. — Zymosan selectively inactivates the thermostable third component (C′3) of complement (1). A rheumatoid pooled serum was treated with zymosan. The complement lytic activity was destroyed by this treatment, but no change occurred in the AAF activity of this serum.

TABLE 2

FRACTIONATION OF RHEUMATOID SERA BY CO_2 DILUTION METHOD

Rheumatoid Serum	Treatment Subjected to	Aggl. Titer for Sensitized Cells	Complement Activity
»Va» (individual)	Whole serum, fresh		Total hemolysis
»	» » inactivated at 56° C	2560	None
»	Precipitate fraction	640	»
»	» » , ammonia treat.	640	»
»	Supernatant fraction	320	»
»	Supernatant fraction, ammonia treat.	160	»
»	Supernatant and precipitate recombined	640 [1]	Total hemolysis
»	Supernatant and precipitate, recombined, ammonia treated	160	None
»	Supernatant and precipitate, recombined, inactiv. at 56° C	640	»
»R—1» (pool)	Whole serum, inactivated at 56° C	5120	None
»	Precipitate fraction	1280	»
»	» » , ammonia treated	640	»
»	Supernatant fraction	320	»
»	» » , ammonia treated	320	»
»Pal» (individual)	Whole serum, three days in icebox	5120	Trace of hemolysis
»	» » , inactivated at 56° C	5120	None
»	Precipitate fraction	2530	»
»	» » , ammonia treated	1280	»
	Supernatant fraction	320	»
	» » , ammonia treated	320	»

None of the above preparations agglutinated non-sensitized cells in a dilution $\geqq 1:40$.

[1] In the first three tubes of the dilution series (up to dilution 1: 160), a decreasing hemolytic effect was noticeable.

COMMENT

The results of the experiments described did not give support to the assumption that the agglutination activating factor (AAF) present in many rheumatoid sera would be identical to or closely associated with the thermostable fourth component of complement

(C′ 4), as suggested by Gorrill and Hobson, or with any other single component of complement. The reason for this discrepancy between our results and those of the above authors is unknown.

SUMMARY

The suggested association of the agglutination activating factor (AAF) peculiar to many rheumatoid sera with the thermostable fourth component of complement (C′4) is investigated. The data obtained do not lend confirmatory evidence to the supposed existence of such a relationship.

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TETRAZOLIUM TEST WITH GERMICIDES

by

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Kuhn and Jerchel (9) showed that the viability of bacterial cells is indicated by tetrazolium salts. According to Wallhäusser (12), this phenomenon can be used in evaluating antibiotic and disinfecting agents. Since the determinations of the phenol coefficients with quaternary ammonium compounds have shown inaccuracies with the usual methods (2, 6), we have tried to verify the usefulness of the tetrazolium test in comparative evaluations of these compounds and phenol.

In all the experiments the test organism was a strain of *Proteus vulgaris* isolated from a routine sample. It stained intensively red in media containing tetrazolium salts; the dye was easily extracted from it with acetone.

The germicides investigated were: commercial Amisept (Lääke Oy) commercial Sterilan (Oy Medica Ab) and commercial Desivon (Astra), all 10 per cent watery solutions of alkyl-dimethyl-benzyl-ammoniumchloride; commercial Bradosol (Ciba) β-phenoxyethyl-dimethyl-dodecyl-ammoniumbromide; commercial Roccal (Winthrop) 10 per cent watery solution of a mixture of alkyl-dimethyl-benzyl-ammoniumchlorides; commercial Desivon (I.C.I.) 20 per cent watery solution of cetyl-trimethyl-ammoniumbromida; Propamidine (May and Baker) 4: 4' diamidino-diphenoxypropane and watery solution made from liquefied phenol.

Method. — The test organism was grown for 4 hours in broth with 10 per cent horse serum added. One cc of the culture was inoculated in plain nutrient broth in bottles containing 500—1000 ml and was

incubated for 24 hours at 37° C in a thermostate. Two cc of the culture was pipetted in tubes with inner diametre of 15 mm and 0.5 cc of germicide solution in water (in controls sterile distilled water or sterile saline was used) and some crystals of glucose were added to stimulate the metabolism (11). The content of tubes was carefully mixed and the tubes were left at room temperature for five minutes (if not otherwise reported). Thereafter 0.5 cc of 0.5 per cent triphenyl-tetrazoliumchloride was added, the content was mixed and the tubes were kept at 37° C in a water bath for 10 minutes. 0.5 cc of acetone was added to extract the dye from the cells, the tubes were shaken vigorously and were allowed to stay at room temperature for 15 minutes. Two cc. of n-butanole was the added and the tubes were shaken in a Kahn-shaker until all the dye was dissolved in butanole (when the layers were allowed to settle, no colour could be seen with naked eyes in the lower part). The intensity of the colour was estimated with EEL-photoelectric colorimeter with filter No 625 and n-butanol as a standard reading 0.

Control Experiments. — A. — Two cc of a culture in plain

TABLE 1

DETERMINATION OF THE STANDARD DEVIATION AND STANDARD ERROR

Bottle I	Bottle II	Bottle III		Bottle IV		
1: 1	1: 1	1: 1	2: 3	1: 1	1: 2	2: 3
63	62	45	18.5	30	17.5	7
75	68	53	15.5	32	18	7
72	60	45	16	32.5	18	8
68	58	47	18	34	18	8
72	62	46	18	34.5	19	8.5
68	64	53	15	35	20	8.5
77	59	49	17	36	20	8.5
72	55	47	16	36.5	20.5	8.5
—	64	44	16	38	21	10
—	—	49	—	35	22	11
70.87	61.33	47.8	16.6	34.35	19.4	8.55
±4.69	±3.84	±3.00	±1.25	±2.22	±2.19	±1.32
s=4.42	s=3.84	s=3.19	s=1.25	s=2.34	s=2.32	s=1.34
σ=1.56	σ=1.28	σ=1.00	σ=0.42	=0.74	σ=0.73	σ=0.44

$$s=\frac{\sqrt{\sum(x_i-\bar{x})^2}}{n-1} \qquad \sigma=\frac{s}{\sqrt{n}}$$

nutrient broth, dilution of 2: 3 or 1: 2 with sterile saline were pipetted in each of the ten tubes, 0.5 cc sterile saline and some glucose crystals were added and the tubes were kept at room temperature for five minutes before the tetrazolium test was performed. In addition, some other cultures were tested. The results are given in Table 1.

B. — The broth culture was diluted with sterile saline in following proportions: 10: 10, 9: 10, 8: 10, 7: 10, 6: 10, 5: 10, 4: 10, 3: 10, 2: 10, and 1: 10. The density of dilutions was estimated with nephelometer and tetrazolium test was performed with the same dilutions. The results are given in Figure 1.

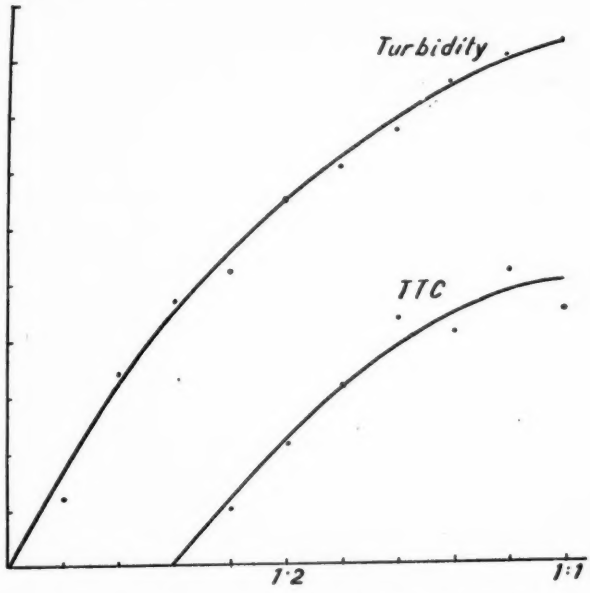

Fig. 1. — Diagram showing the curves of turbidity and TTC values of bacterial dilutions.

C. — The growth curve was followed. One cc of 4 hours culture of broth with 10 per cent horse serum was inoculated in 1000 ml of plain broth and incubated at 37° C in a thermostate. Samples of two cc were taken after the bottle was shaken, after 1, 2, 4, 6, 8, 9, 10, 11, 12, and 24 hours incubation time. The density was estimated with nephelometer and the tetrazolium test was performed from the same samples. In addition, the number of bacteria was es-

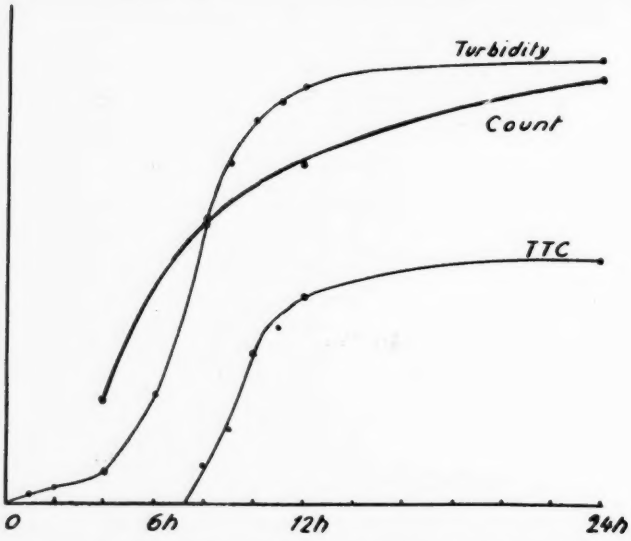

Fig. 2. — Diagram showing the growth curves established with nephelometry, bacterial count and TTC-estimation.

timated after 4, 8, 12 and 24 hours incubation with membrane filter technique (8). The results are given in Figure 2.

We can see from the foregoing experiments that the stronger the colour intensity, the greater the standard deviation and mean error. This fact may be caused by the logaritme scale of the colorimeter. On the other hand, both the deviation and the error are insignificant according to the biological method. We note further that the colour intensity is directly dependent of the number of the living bacteria, however, not linearly.

Effect of the Germicides on Bacteria. — It was estimated in the preliminary experiments with ten-fold dilutions of the germicides that with the following concentrations the colour intensity readings were approximately one half of the intensity of the conrols:

Phenol	.....	0.18 per cent
Amisept	.....	0.002 » »
Sterilan	.....	0.002 » »
Desivon	.....	0.002 » »
Bradosol	.....	0.002 » »
Roccal	.....	0.002 » »
Cetavlon	.....	0.01 » »
Propamidine	.....	0.02 » »

TABLE 2

THE COLORIMETRIC VALUES FOR THE COLOUR INTENSITY WHEN GERMICIDES WERE ADDED IN DIFFERENT CONCENTRATIONS

Germicide Dilutions							
Phenol bacterial dilution	0.18 %	0.09 %	0.045 %	0.0225 %	0.0112 %	Controls	
1: 1	13	30	50	38	38	57	62
2: 3	5		19			35	30
1: 2	2.5		7			16	15
Amisept	0.002 %	0.001 %	0.0005 %	0.00025 %	0.00012 %		
1: 1	5	34	34	50	78	80	84
2: 3	0		15			54	53
1: 2	0		11			35	40
Sterilan	0.002 %	0.001 %	0.0005 %	0.00025 %	0.00012 %		
1: 1	13	29	33	42	80	80	82
2: 3	3		19			44	54
1: 2	0		12			34	34
Desivon	0.002 %	0.001 %	0.0005 %	0.00025 %	0.00012 %		
1: 1	17	34	35	48	90	105	115
2: 3	0		15				70
1: 2	0		15			44	44
Bradosol	0.002 %	0.001 %	0.0005 %	0.00025 %	0.00012 %		
1: 1	1	33	31	41	80	100	100
2: 3	0		20			66	66
1: 2	0		15			40	37
Roccal	0.002 %	0.001 %	0.0005 %	0.00025 %	0.00012 %		
1: 1	1	18	18	28	60	62	69
2: 3	0		10			35	42
1: 2	0		8			26	29
Cetavlon	0.01 %	0.005 %	0.0025 %	0.00125 %	0.0006 %		
1: 1	0	0	12	23	40	58	50
2: 3	0		4			31	33
1: 2	0		0			18	17
Propamidine	0.02 %	0.01 %	0.005 %	0.0025 %	0.00125 %		
1: 1	8	16	23	48	62	73	72
2: 3	4		13			35	42
1: 2	2		8			26	29

Controls with Two-fold Bacterial Dilutions			
75	18	2.5	0
80	26	1	0
54	15	3	0

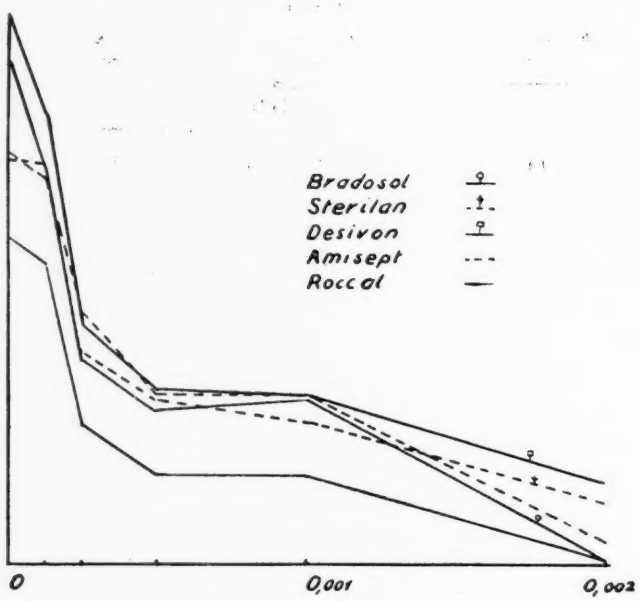

Fig. 3. — Diagram showing the TTC estimations of quaternary ammonium germicides effect on bacterial populations.

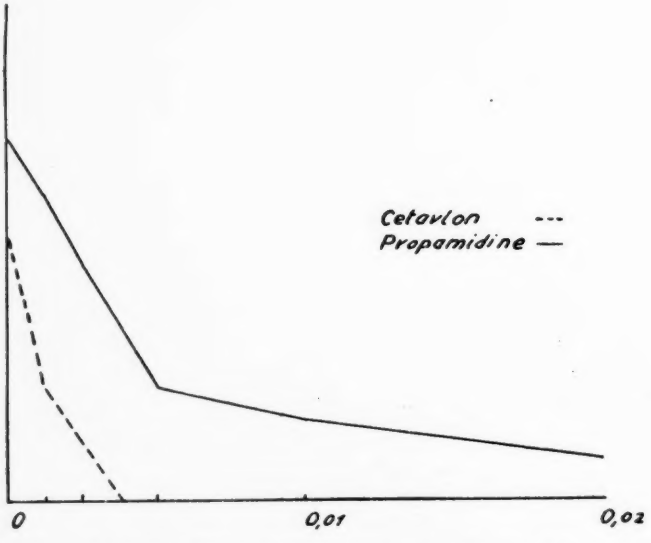

Fig. 4. — Diagram showing the TTC estimations of Cetavlon and Propamidine effects on bacterial populations.

Accordingly, these bacteriostatically roughly equivalent concentrations were selected for the following experiments.

The effect of germicides was determined with two-fold dilutions on a) an undiluted culture, b) a culture diluted 2: 3 with sterile saline and c) a culture diluted 1: 2 with sterile saline. Two tubes with sterile distilled water (instead of germicide) were used as controls in every series. From every bottle a two-fold dilution of bacteria was tested as an additional control. The results are given in Table 2 and in Figures 3 and 4.

Two series starting with 0.002 per cent Cetavlon were also performed, as the concentration 0.01 inhibited the colour formation. The results are given in Figure 5.

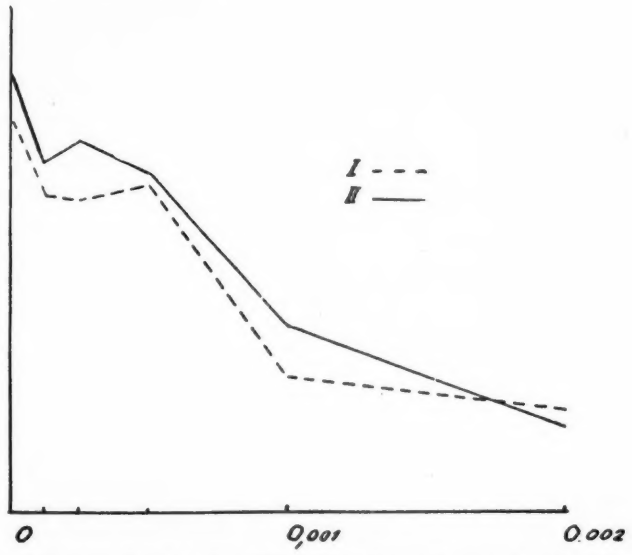

Fig. 5. — Diagram showing the TTC estimations of two tests with Cetavlon.

The Time-Factor. — Concentrations n (= equivalent concentration) n/4 and n/16 were chosen from the previous experiments and new series were performed. At room temperature before the addition of tetrazolium the activity times were varied between 5, 10, 15 and 30 minutes. Controls were similar to those used earlier. The results are given in Table 3 and Figures 6, 7 and 8.

Additional Tests with Phenol. — Since some »prozone trends» were seen in the more diluted parts of the effectivity curve of phenol

TABLE 3

EFFECT OF THE ACTIVITY TIME ON THE COLORIMETRIC VALUES

	Phenol	0.18 %	0.045 %	0.001 %	Controls		Amisept	0.002 %	0.0005 %	0.0001 %	Controls	
5′		16.5	58	58	63	75		19.5	32.5	72.5	73	85
10′		16	60	61	72	68		21	41	76	85	80
15′		12	43	53	62	68		17	57	80	90	84
30′		8.5	42.5	50.5	60	58		13.5	45.5	76	87	87
	Sterilan	0.002 %	0.0005 %	0.0001 %			Desivon	0.002 %	0.0005 %	0.0001 %		
5′		8.5	33	45	72	68		1	24	61.5	74	72
10′		13.5	32	68	77	72		0	38	61	70	68
15′		6.5	19	45	62	64		1	25	59	75	68
30′		3	18.5	39.5	59	55		1	24	61	72	74
	Bradosol	0.002 %	0.0005 %	0.0001 %			Roccal	0.002 %	0.0005 %	0.0001 %		
5′		0	16.5	53	70	77		2.5	14	29	45	60
10′		0	15	62	76	69		2.5	12.5	34	48	63
15′		0	17	65	69	81		3.5	18.5	46	69	79
30′		2	22	59	61	62		0.5	11	26	55	68
	Cetavlon	0.01 %	0.0025 %	0.0006 %			Propa-midine	0.02 %	0.005 %	0.0012 %		
5′		0.5	20	65	79	78		10	34	41	68	62
10′		1.5	18	54	82	72		6	33	43	55	61
15′		0	19	64	81	77		7	23.5	59	72	73
30′		0	17.5	59.5	72	72		11	22	39.5	48	67

Controls with Two-fold Bacterial Dilutions			
43.5	9.5	1	0
64	17	0	0
47	12	0	0
56	14.5	0	0
45	15	1	0

and Cetavlon, the tests with phenol were repeated at tighter intervals in concentrations to eliminate the occasional technical error. The results are given in Table 4. The average curve calculated from these results is shown in Figure 9.

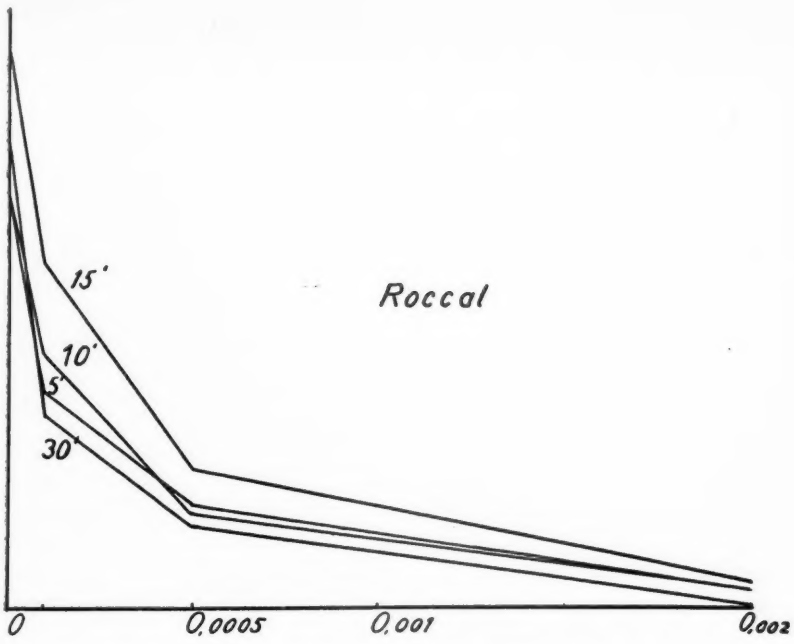

Fig. 6. — Diagram showing the TTC estimations with different activity times with Roccal.

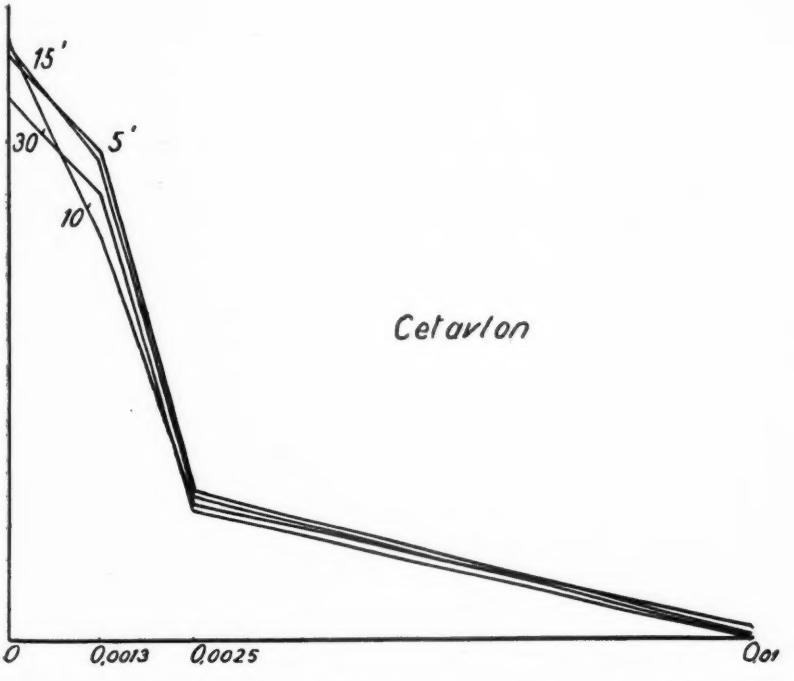

Fig. 7. — Diagram showing the TTC estimations with different activity times with Cetavlon.

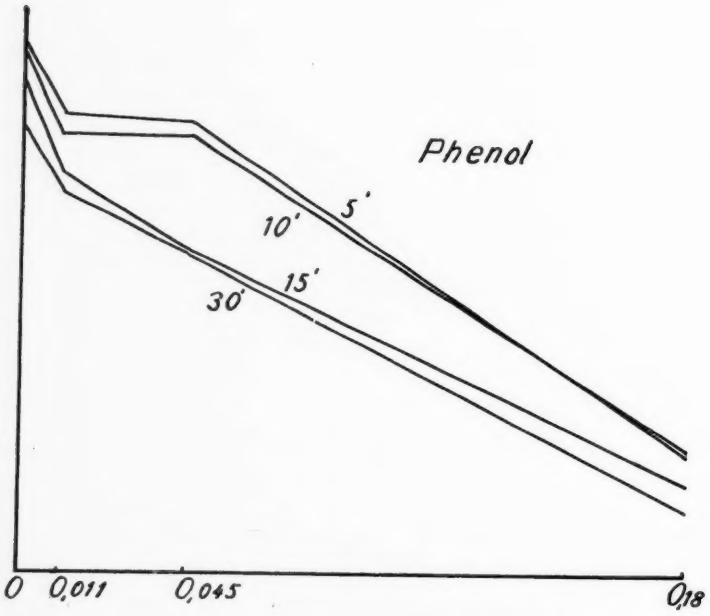

Fig. 8. — Diagram showing the TTC estimations with different activity times with phenol.

TABLE 4

COLORIMETRIC VALUES WITH PHENOL IN SLIGHT DILUTION DIFFERENCES

Dilutions per Cent												Control
0.18	0.144	0.108	0.09	0.072	0.063	0.054	0.045	0.036	0.027	0.018	0.009	
6	7	5	×	3	10	25	40	34	31	60	83	83
7	10	10	×	16	10	17	25	15	5	39	63	87
13	16	17	×	20	10	13	37	25	31	33	63	67
4	4	4	×	4	7	10	20	27	31	55	57	76
20	20	17	38	×	34	40	52	56	62	51	64	68
10	13	15	11	×	28	43	47	47	56	58	57	63
12	11	15	13	×	13	27	38	37	45	30	55	61
7	10	7	16	×	30	41	39	46	48	56	53	59
8	7	9	13	×	9	29	28	28	30	28	42	58
5	8	7	13	×	14	19	16	17	21	26	32	40
7	9	9	9	×	16	14	15	33	34	28	27	36

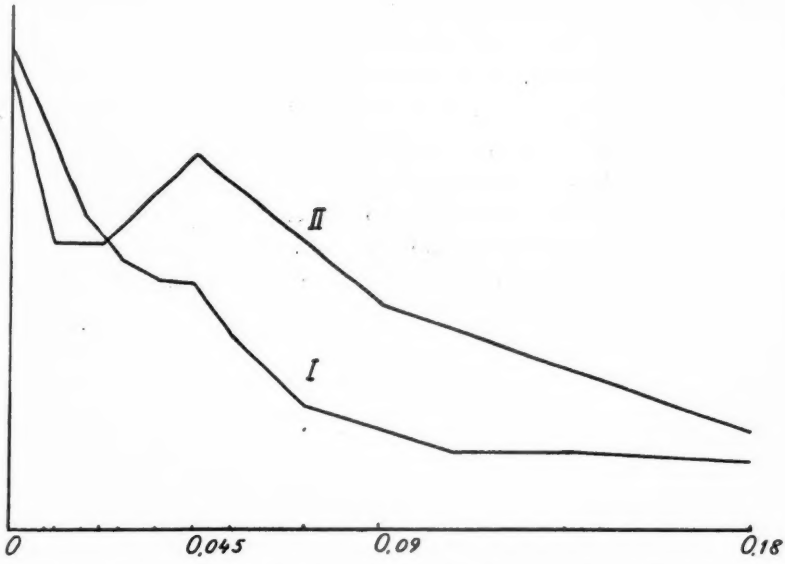

Fig. 9. — Diagram showing the average effectivity curve I and the primary single effectivity curve II of phenol dilutions. For explanations see text.

DISCUSSION

The tetrazolium salts *per se* can be bacteriostatic or bactericidal in sufficient concentrations. The concentration used (0.083 per cent) was, however, optimal for both the growth and colour production of this test organism (13). To what extent it can prevent the metabolism of bacteria, »weakened» by germicides, could not be established. As the tetrazolium test measures only the reduction ability which can be retained by cells no longer capable of multiplying (5) the test can also in this way give inaccurate results. This must be kept in mind in determinations with phenol, especially, which only adsorbs to the bacterial cell, but does not penetrate the cell wall (3), particularly in diluted concentrations. In spite of the most definite stability in the growth conditions, considerable variation in colour intensity in controls could be noted. The tests were performed on various instants of the day and the weakest colours were obtained in series made in dark hours. According to Jambor (7), the reduction depends not only on the enzymatic action of bacteria, but also on the luminousness. Therefore »conditions must be carefully controlled when tetrazolium is used for quantitative experiments».

When tetrazolium test is used to establish the effectiveness of germicides, we cannot apply the same principles as in usual technics of phenol coefficient. Small numbers of bacteria do not give noticeable changes in colour. The sensitivity of the test lies in about 1—10 million organisms per milliliter, dependent on the luminousness. Accordingly we can only determine a rough relative effectiveness on great number of organisms with a germicide concentration, which kills but a part of the organisms. However, if we calculate the proportion of the dilutions of the different germicides to the dilutions of phenol which give corresponding colour intensity (in series with the same colour intensity in controls), we see that Propamidine has about 36 times, Cetavlon about 72 times and the others about 360 times greater effectiveness than phenol. The comparison of these results with phenol coefficients established otherwise shows that they do not strikingly differ from some other results (10). Because of the differences in controls, we can not compare the results of different experiments. Even if we do not get absolute inhibition values, we can see, that Bradosol, for instance, is somewhat more effective than Desivon.

In experiments with different activity times it would have been justified to use some kind of inactivator before the addition of tetrazolium, to establish the real time of activity. In the technic used the germicide was continuously killing the bacteria during the formazan formation in the water bath. However, as we had no substance, which could inactivate both the quaternary ammonium compounds and phenol (1), we could not use any universal inactivator. In the light of the results there was no reason to use any inactivator. It is known that cetrimide (= Cetavlon) (4) in a concentration of 0.002 per cent disintegrates about 99 per cent of bacteria in five minutes, while with tetrazolium test Cetavlon, with a concentration of 0.0025 per cent, produced only about 50 per cent diminution in colour intensity in five minutes activity time, and the lengthening of the time did not increase the effect significantly. Accordingly the tetrazolium test establishes here only the enzymatic reduction activity of bacteria without ability to multiply. As the reduction activity is not altered with a prolonged action of germicides used, the activity time is not a decisive fastor in the test.

In additional tests with phenol we noted values suggesting a prozone phenomenon in almost every series. This could not be seen

in any other germicide except Cetavlon. Anyhow, no deviation occurs in the average curve calculated from these additional tests to verify the phenomenon statistically. The problem of the prozone phenomenon needs further investigation.

The following requirements are presented to the germicide evaluations (6): 1. simplicity, 2. inexpensiveness, 3. accuracy, 4. reproducibility, 5. usefulness over a range, and 6. relation to the service the product is to perform. The first two requirements are fulfilled with tetrazolium test, the fourth may be fulfilled with stabilized conditions and the fifth seems to be fulfilled. On the contrary, the accuracy leaves room for criticism. So even though the tetrazolium test gives a new principle to the evaluation of the affect of germicides on great populations, it is questionable, if the test is serviceable to the quantitative classical evaluation of germicides.

SUMMARY

1. The effect of substerilizing concentrations of germicides on great populations of bacteria can be established with the tetrazolium test.

2. The time of activity is not a decisive factor in the test, since it gives only the reduction ability of cells.

3. The accuracy of the test is not great, but it gives, anyhow, results comparable with some other methods.

4. The conditions should be carefully controlled when using the test.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

THE ACTION OF PROPERDIN ON TOXOPLASMA GONDII

A PRELIMINARY REPORT

by

PAUL GRÖNROOS

(Received for publication January 11, 1955)

The mechanism of the Sabin-Feldman dye test is still obscure, in spite of, or perhaps because of, the fact that the phenomenon involved is unique in serology (7). It seems unlikely that the so-called activator component should block receptors for methylene blue and that Toxoplasma should, therefore, remain unstained. Bringmann and Holz are against this theory and base their opinion on electron microscopic studies of Toxoplasma stained and unstained in the dye test. They remark that ribonucleic acids of cytoplasm are released from the unstained Toxoplasma and that the Toxoplasma cannot be stained for this reason (1).

A. — With regard to the mechanism of the dye test, Jettmar's preliminary report on antitoxoplasmic properties in human serum is of considerable interest. He discovered in normal serum a »Toxoplasma-hostile» component that was inactivated at + 56° C in as short a time as 15 min. This component was not usually found in children. It can be demonstrated in certain sera to the titer 1/64. Among various animal sera, the bovine sera are abundant in this component (2).

The author performed experiments on »Jettmar's factor», using a technique similar to that of Jettmar. The changes in Toxoplasma cytoplasm obtained by Jettmar's factor are, in general,

so pronounced that it is easy to divide the Toxoplasma visible microscopically into irreversibly affected, *i.e.*, positive, and non-affected, *i.e.*, negative, organisms. The affected positive Toxoplasma are usually somewhat rounder, and the cytoplasm appears to be highly disintegrated and granulous under the phase contrast microscope. The »color»[1] of the cytoplasm is rather light gray, except for that of the granules which can be markedly bluish. Particles attached to the surface of positive Toxoplasma, can often be noticed; they are derived either from the surrounding liquid or from the affected Toxoplasma themselves. When studied under Wild's Varicolor microscope, some of the positive Toxoplasma seem to be filled with a granulous mass. Under this microscope a broad clear halo can be seen distinctly around the negative Toxoplasma. The halo is especially apparent in slightly red light. The negative Toxoplasma are a beautiful uniformly grayish blue, without any pronounced granules. The halo is hardly visible around the positive Toxoplasma and argues in favor of its diminished optical density (due to loss of substance?). These observations seem to support Bringmann and Holz's concepts based on electron microscope studies.

In the present work, the Toxoplasma-hostile factor was studied in its relation to properdin. As a typical example of changes in serum after Zymosan[2] treatment can be mentioned serum $k_{30}I$, which was investigated a few hours after it was taken. See Table 1.

The results obtained with serum k_{30} suggested that the Toxoplasma-hostile factor is lacking after serum is treated with Zymosan. Zymosan was added in the amount of 5 mg/ml of serum. As Pillemer and co-workers (3) pointed out, Zymosan removes a factor they called properdin. We know further that treatment of serum with Zymosan at 15—18° C for 75 min. removes properdin almost exclusively, while at a temperature over 20° C the complement factor C_3 can also be removed. It can be seen from Table 1 that a decisive role of C_3 in the degenerative changes in Toxoplasma cytoplasm is hardly possible. To show further that properdin is the more important factor, a two-step removal of properdin was performed according to Pillemer. »A single treatment of serum with

[1] The »color» is defined according to Pulvertaft *et al.* as follows: »The appearance seen once only on varying the focus is the correct appearance.»

[2] Zymosan was prepared from Finnish press-yeast according to Pillemer (4).

TABLE 1

THE ACTION OF ZYMOSAN ON DIFFERENT SERA

	$K_{30}I$	$K_{30}II$	$Å_{26}I$	$Å_{26}II$	T_2I	T_2II
Buffer pH 7.4 (containing Mg.)	8	12	16	10	10	16
Serum 1/1	86	90	94	90	96	94
1/2	52	78	50		94	90
1/4	28	40	26		96	86
1/8					76	82
1/16					52	
Serum treated with Zymosan 75 min. at 18° C before the »Jettmar test»	24		90			
Serum treated with Zymosan for 75 min. at 37° C before the »Jettmar test»	22					
Serum held at 37° C for 75 min. without Zymosan	78		90	78	98	
Serum treated by the »two step» method (see text)		20	70	8	80	96
Serum inactivated at 56° C for 30 min.	6	16	6		12	8

Figures in Table 1 give the number of positive lysed cells out of a hundred counted toxoplasma. The serum dilution was 1/1, if not otherwise stated.

Zymosan at 15° C to 18° C is often insufficient to remove all the properdin. Such a serum may be used for the assay of properdin, but it is not always suitable for bactericidal and virus-neutralizing studies. A second treatment of the serum with Zymosan at 37° C for 15 to 30 min. removes all the remaining properdin with only a slight loss of [complement] C' or C'-component activities» (3). Serum k_{30} was treated in this way, and the results are shown in Table 1 (k_{30} II). The use of this serum, which should contain C_3, together with the guinea pig complement from which C_3 was removed with previous Zymosan treatment at 37° C for 75 min., and which, as such, was ineffective as a complement, made it possible to re-establish the complement activity of this guinea pig complement almost entirely. This supports the fact that properdin is an essential factor in the development of degenerative changes in Toxoplasma cytoplasm. According to Jettmar, bovine sera contain great amounts of the Toxoplasma-hostile factor. Pillemer reported that cattle

serum contains 10 to 20 units of properdin per ml (human serum contains 4 to 8 units per ml).

The afore-mentioned serum k_{30} reaction type was the most common among the sera studied. The highest titer found (measured according to Jettmar) was 1/16. In a smaller number of sera it was impossible to remove the Toxoplasma-hostile factor (properdin?) entirely. Sera $Å_{26}$ and T_2 (see Table 1) were of this type. After storage for some time at —14° C, the Toxoplasma-producing factor could be entirely removed from $Å_{26}$ (see Table 1, $Å_{26}$ II), but not from T_2 (see T_2 II). Studies are in progress on the significance of temperature during this phenomenon.

B. — Sera $Å_{26}$ and T_2 were dye test positive, and it was thus thought justified to use them in studying the role of properdin in the dye test. A non-treated activator serum and a serum treated with the two-step system gave the results shown in Table 2 on the dye test with a known strongly positive Toxoplasma-immune serum (dye test titer, 1/16,386).

TABLE 2

THE ACTION OF ZYMOSAN ON THE ACTIVATOR IN THE DYE TEST

Activator Serum	Immune Serum Dilutions			
	1/4	1/16	1/64	1/256
Non-treated serum	96	94	94	88
Serum treated with two-step system	22	16	16	10

Figures in Table 2 indicate the percentage of the unstained Toxoplasma in the respective dilutions (dye test).

Properdin is, accordingly, an important factor in activator serum. Sabin and Feldman (7) were of the opinion that the activator serum is much like the usual guinea pig complement (C_1, C_2, C_3 and C_4) without, however, being identical to it. Roth (6) remarked that the activator serum has C_2, C_3 and C_4 in common with the usual guinea pig complement. According to Roth, C_1 is not necessary, while, on the other hand, an unknown factor is needed which is removed together with C_3 on yeast treatment of serum. On the basis of the afore-mentioned observations, we can draw the following conclusion:

Activator serum = Properdin $+C_2 + C_3 + C_4$.

DISCUSSION

Results obtained on Zymosan treatment of serum $Å_{26}$ before and after storage suggested that a specific antibody *in vivo* is associated with properdin. The fixation point of Toxoplasma antibody on the properdin molecule could be thought to be the same as that of Zymosan. In this way the Zymosan-accepting receptor is blocked. It will be of interest to note whether binding of similar type will occur with other antibodies.

The changes in Toxoplasma that properdin produced alone and together with immune serum (as measured by the dye test), are surprisingly analogous. If we stain Toxoplasma that are affected only by properdin with methylene blue, we obtain positive and negative Toxoplasma exactly as in the dye test. It seems likely that properdin (perhaps together with C_2, C_3 and C_4) has a capacity equal to that of properdin and immune serum together on a larger scale. The final mechanism is still obscure, but the following hypothesis might be taken into consideration. Properdin is a non-specific »antibody» acting on the surface of other cells and forming a lytic system with C_2, C_3 and C_4. This system acts upon the cells' surface so that specific antibodies can influence the inner parts of the cells. If the properdin content of serum is large enough, the properdin system alone can alter the cell surface so that the cells are irreversibly damaged.

It is possible, however, that there exist two Toxoplasma-damaging systems with parallel effects. The other immune system acts at even greater dilutions. Thus, the role of properdin and a specific antibody would be to combine the lytic system with the cell surface. We must keep in mind, on the other hand, that the dye test does not function without properdin. It is further obscure whether properdin can combine non-specific antibodies with Toxoplasma. It can be assumed that properdin may act as an intermediary between, *e.g.*, Sarcocystis or Trichomonas antibodies and Toxoplasma.

It is clear, at any rate, that the properdin content, if sufficient, can act upon Toxoplasma so that a subsequent methylene blue stain gives a picture similar to that obtained on the dye test. It is thereby necessary to inactivate the serum at 56° C for 30 min.

Still the questions arise whether the dye test is really specific

for Toxoplasma and what we actually measure with it. In any case, the afore-mentioned results suggested a serologic and nonspecific mechanism that may also occur in the dye test. In addition, the properdin content of serum increases with increasing age.

Toxoplasmosis congenita is a relatively rare disease. It may be assumed that the fetal infection becomes evident only when the mother's properdin content is low and the specific antibodies have not yet developed.

Jettmar's method seems to be useful in determining the properdin content of serum, at least in dye test negative serum. The method has perhaps no advantages in comparison with that reported by Pillemer, on the contrary, there occurs the danger of laboratory infection. There is, indeed, a possibility that Zymosan is not always able to precipitate properdin (serum $Å_{26}$).

SUMMARY

1. Properdin has a lytic effect upon Toxoplasma.

2. The activator serum in the Sabin-Feldman dye test seems to be = properdin $+C_2 + C_3 + C_4$.

3. It is possible that properdin can give nonspecific positive results in the dye test.

4. A theory on properdin's role in the dye test is presented.

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FROM THE STATE SERUM INSTITUTE, HELSINKI

SERUM CONCENTRATION OF DIFFERENT COMMERCIAL PENICILLIN PREPARATIONS

III

PENICILLIN TABLETS

by

TAPIO SAVOLAINEN and VEIKKO TOMMILA

(Received for publication January 18, 1955)

PRESENT TESTS WITH PENICILLIN TABLETS

We have determined the serum penicillin concentrations attainable in adult hospital patients during six hours after administration of some commercial penicillin tablets.

The penicillin dosage was in all cases 600.000 units, corresponding to three tablets of 200.000 units of procaine penicillin G. The tablets were given in the morning to fasting patients, and blood samples were drawn just prior to, and 1, 2, 3 and 6 hours after administration.

The technique earlier described (5, 7) was used in the serum penicillin determinations. The test subjects were adults hospitalised in the Helsinki University Hospital for Ear, Nose and Throat Diseases.[1] Four different procaine penicillin tablets made by different manufacturers were procured in the manner reported earlier (4, 5, 6). Each product was tested on a series of five patients. The age of the patients ranged from 17 to 56 years and their body weights from 52 to 75 kg.

[1] We gratefully acknowledge the kindness of Professor Y. Meurman, M.D., Chief of the Helsinki University Hospital for Ear, Nose and Throat Diseases in placing at our disposal a sufficiently large series of patients.

RESULTS

The results are shown in the form of a curve in Fig. 1.

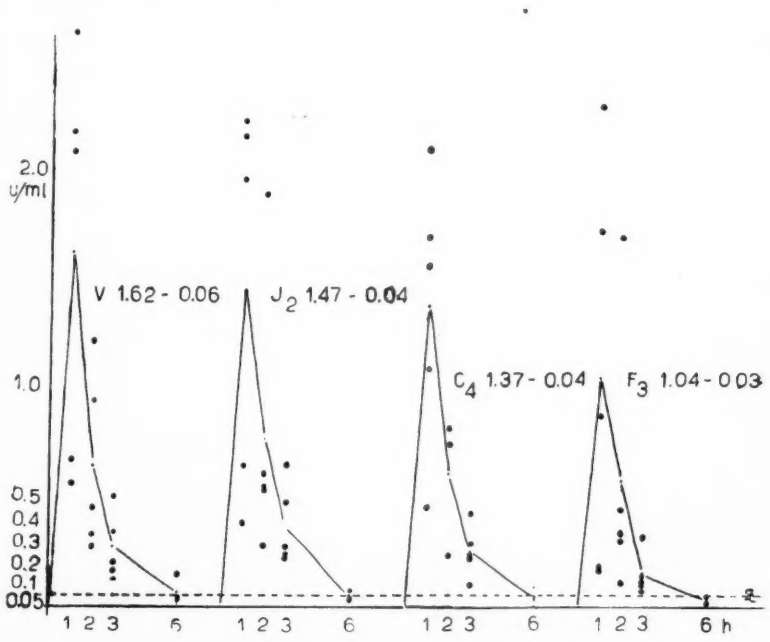

Fig. 1.[1] — Average penicillin concentrations in sera of adults during six hours after a single peroral dose of 600.000 units of four different procaine penicillin G tablet preparations.
z) The minimum therapeutic concentration.

[1] The preparations are designated by letters; small figures indicate the numbers of the products of the same manufacturer. In the following columns are given the mean maximum concentration (in units per ml of serum) obtained with each preparation and the mean concentration (in units per ml of serum) six hours after administration. Dots above and below the curves show the serum penicillin concentrations of each patient at the times stated.

Table 1 shows the standard deviations of the mean values.

TABLE 1 [2]

TESTED PROCAINE PENICILLIN G TABLETS, NUMBER OF PATIENTS, MEAN PENICILLIN CONCENTRATIONS IN SERUM AND STANDARD DEVIATIONS FOR EACH PRODUCT AT MAXIMUM CONCENTRATION AND AT SIX HOURS

Product	No. of Patients	Mean Maximum Concentration		Mean Concentration at 24 Hours	
		u./ml. of Serum	Its Standard Deviation	u./ml. of Serum	Its Standard Deviation
V	5	1.62	±0.42	0.06	±0.02
I_2	5	1.47	±0.40	0.04	±00.1
C_4	5	1.37	±0.28	0.04	±00.1
F_3	5	1.04	±0.42	0.03	±00.1

[2] We are greatly indebted to Mr E. Kaila, Ph.D., for controlling the mean values and calculating the standard deviations.

DISCUSSION

As is seen from Fig. 1, the mean maximum penicillin concentrations attained in the serum with the four tablet preparations were 1.62, 1.47, 1.37 and 1.04 u./ml serum. (The maximum individual values for the different products were 2.62, 2.22, 2.08 and 2.28 u./ml, respectively, and the minimum values *i.e.*, lowest maximum concentrations 0.57, 0.38, 0.45 and 0.17 u./ml, respectively.) Six hours after administration the mean serum penicillin concentrations were 0.06, 0.04, 0.04 and 0.03 u./ml serum.

Thus it may be said that all the preparations tested give relatively high mean penicillin concentrations in the serum and that these mean concentrations were on approximately the same level for all products. However, individual variations in the different patients were great (Fig. 1). This is evident also from the standard deviations of the mean concentrations (Table 1). The mean concentrations for all the products remained above the minimum therapeutic concentration for from 5½ to over six hours. The maximum concentrations were reached rapidly, *i.e.*, within at least one hour, with all the products.

Results similar to those here reported were also obtained earlier by Boger *et al.* (1, 2, 3), although they were able to prolong considerably the duration of the penicillin concentration in the serum by simultaneous administration of penicillin tablets and Benemid.

SUMMARY

1. Using the technique described in a previous report, the serum penicillin concentrations obtained with 600.000 units of four different commercial penicillin tablet preparations were studied in a total series of 20 hospital patients.

2. The maximum concentrations were reached rapidly, *i.e.*, in one hour (with the exception of two cases). The mean maximum concentrations for the different products were of a uniform level and fairly high (*ad* 2.62 u./ml serum).

3. A single dose gave serum penicillin concentrations that remained above the therapeutic minimum concentration for, on an average, 5½ to six hours.

4. Marked individual variations were seen for each product. Thus, for instance, the maximum concentrations for the different products fluctuated between 2.62 and 0.57, 2.22 and 0.38, 2.08 and 0.45, and 2.28 and 0.17 u./ml serum.

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FROM THE INSTITUTE OF OCCUPATIONAL HEALTH, HELSINKI

EFFECT OF FOOD INTAKE ON THE CARDIOVASCULAR RESPONSE TO CIGARETTE SMOKING

by

REINO RUOSTEENOJA

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Smokers are familiar with the phenomenon that tobacco tastes milder and pleasanter after food intake than in the fasting state. The cause of this circumstance has been hitherto unknown. It seemed possible that it would depend on some circulatory changes. It is a fact that tobacco smoking raises the pulse rate (1, 2, 3, 4) as well as the systolic and diastolic blood pressure. These pressure changes are, however, relatively slight (2, 3, 5). Smoking also causes vasoconstriction in the skin of the great majority of people. This has clearly been measured in the peripheral parts of the extremities using skin temperature, plethysmographic and radioactive iodized albumin methods as indicators (3, 5, 6, 7, 8, 9, 10). The above mentioned circulatory changes on smoking after food intake have been examined by Roth (3). Roth claims, however, that the changes are statistically similar in the fasting state and after the meal. But in these examinations the act of smoking was not performed until 1—1.5 hours after eating, i.e. after the vasodilatation induced by the food had clearly taken place. It is true, however, that smoking is especially pleasant immediately after food intake, before any appreciable resorption of food and rise in skin temperature have had time to take place. In this paper, therefore, I intend to investigate what is the reaction of the circulatory system to smoking immediately after a meal compared with that in the fasting state.

METHODS

Ten 20—25-year-old male students were selected as subjects in this study. Half of them were regular smokers, the others smoking only occasionally. Every subject followed a standard smoking scheme in accordance with which one drew a normal inhalation three times a minute during eight minutes. So a Finnish »Boston» cigarette was nearly wholly consumed in every test. All the examinations were performed in the morning and were preceded by a food and tobacco fast of 15 hours. A smoking test without food was first made on every subject, and then on another morning a control test after each subject had taken a substantial meal of pea soup. The tests were performed in a warm room, where the temperature was about 22°C. The subjects lay on a bed for half an hour before and after the beginning of smoking. The basal measurements were taken at the end of the preceding half hour. The pulse rates were measured by palpation of the radial artery. The blood pressures were taken in the upper arm with a sphygmomanometer. The skin temperatures, which are a good estimate of the circulatory state of the skin, were registered by means of a thermocouple gauge in the volar side of the left thumbtip and middle fingertip. The measurements were taken every two minutes during twenty minutes, then later at intervals of five minutes. As an extra control, the corresponding measurements were also taken after a meal without tobacco.

RESULTS

The observations of the last-mentioned control tests showed that in consequence of the meal as such the pulse rate rose by about 5 beats per minute and remained at this level all through the observation period. With regard to the blood pressures no appreciable change was noticed. A decrease of 0.2°C in the skin temperature was measured soon after the meal, but it disappeared in some minutes. Later during the observation time the temperature rose on an average 0.45°C above the original level.

The following facts appeared when the effect of smoking on the vascular reaction was compared in fasting and after food intake. The pulse rate was augmented on an average 22.5 beats per minute in the fasting state and 15.2/min after the meal. The maximal rise took place in the end phase of smoking. The difference between

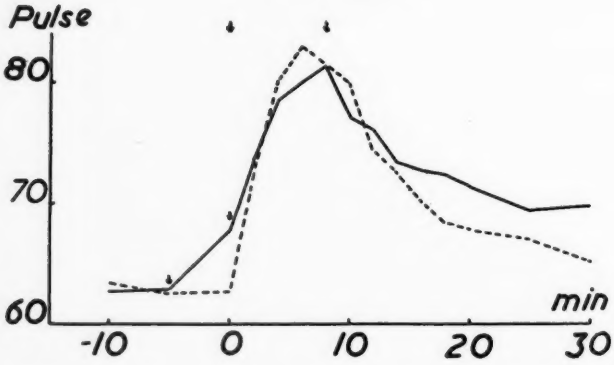

Fig. 1. — The broken line shows the average pulse rates in the fasting state, the solid line in connection with the meal. The meal period was just before the 0-point of the time scale and the smoking period immediately after it. The arrows show the limits of both periods.

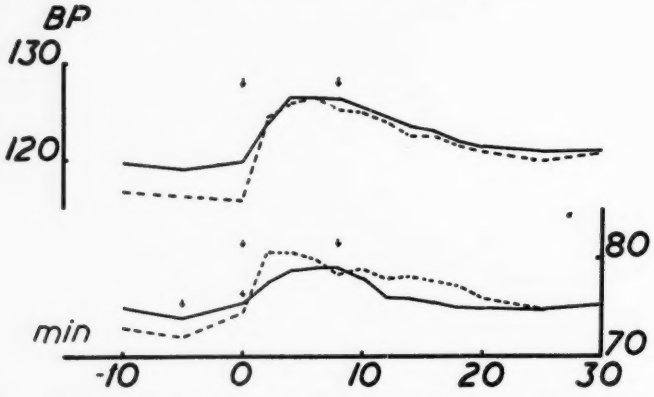

Fig. 2. — The average systolic and diastolic blood pressures expressed as in Fig. 1.

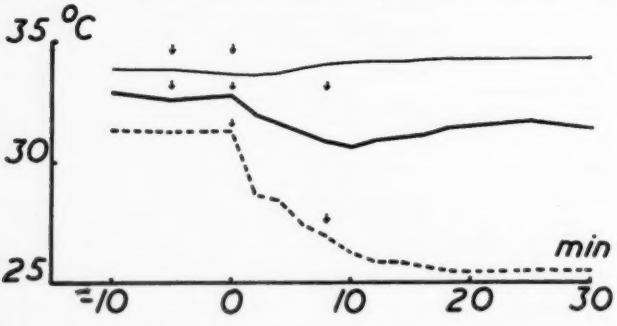

Fig. 3. — The changes in the average skin temperatures of the fingers in connection with the meal as such (thin line), with smoking in the fasting state (broken line) and with smoking after the meal (thick line). The arrows as in the other figures.

these values, 7.3 ± 3.0/min, is statistically just on the limit of significance ($p < 0.05$). Fig. 1 shows the curves of the mean pulse rates. The fasting pulse curve is broken, the curve after the meal is solid. They are so similar that the difference naturally cannot be much appreciated.

On smoking in the fasting state the systolic blood pressure rose on the average 13.0 mm Hg and after the meal the increase was 8.6 mm Hg. Their difference, 4.4 ± 1.8 mm Hg is also scarcely significant ($p < 0.05$). The corresponding values in the diastolic pressures were 8.1 and 5.8 mm Hg. Their difference, 2.3 ± 1.0 mm Hg is significant to the same degree ($p < 0.05$). Here also the maximal rise was observed at the end of smoking. Fig. 2 shows the blood pressure curves corresponding to the former figure. They are still more like each other.

The measurements of skin temperature gave much clearer differences. When smoking took place on an empty stomach, the finger temperature diminished by 6.4°C, but after a meal the decrease was only 2.8°C. This difference, $3.6 \pm 0.8°$, is highly significant ($p < 0.001$). As becomes apparent from Fig. 3 a sharp drop in temperature took place in the first case during twelve minutes after eating (broken line). Then quite a slow decrease continued to the end of the observation period. Smoking after the meal diminished the temperature somewhat during ten minutes from the beginning of smoking, but then, vice versa, a slow rise began to take place. In the Fig. 3 there is also a thin line drawn above the other lines, which shows the relative changes in skin temperature induced by the meal as such.

DISCUSSION

Regarding the results it may be said that the differences in the rise of pulse rates and especially blood pressures are too small to allow an adequate evaluation of their statistical significance. Thus the cardiac output on smoking is nearly as great in fasting as after the meal. But on the contrary the effect of smoking is clearly stronger on the peripheral cutaneous circulation in the fasting state than immediately after food intake. This difference is so great that it is not at all accounted for by the small dilatatory influence due to the meal as such. What is the reason for this different reaction?

It is only possible to put forward some hypotheses. As is known, nicotine has a very complicated action on the vegetative nervous system. It both stimulates and blocks different parts of this system. It has been claimed that the effect of smoking on the circulation partly takes place through the release of adrenaline from the adrenal medulla (2, 11). Nicotine and agents released by it might be more effectively inactivated, when the splanchnic vessels are dilated after the meal and a higher proportion of the blood flows through the liver. If any of the nicotine released during smoking enters the circulation after having been swallowed, it is obvious that a full stomach will retard its resorption.

As, in any case, there seems to be some evidence that smoking of tobacco is a contributory factor in the etiology of coronary disease and thromboangiitis obliterans (3, 12, 13), it might be well-founded to take these results into consideration in clinical practice. Thus patients disposed to these diseases ought to avoid smoking in the morning before breakfast, because the first nicotine dose of the day has the strongest vasoconstrictory effect. Later in the day, habitual smokers have a constant moderate vasoconstriction and so the changes caused by smoking are smaller (3).

SUMMARY

The experiments were performed in the morning after 15 hours' abstinence from food and tobacco. Ten subjects — 5 smokers and 5 non-smokers — were let to smoke a cigarette immediately after a pea soup meal and as a control series without food intake. The pulse rate, the blood pressure and the skin temperature in fingers were measured.

The increase in pulse rate was 7.3 ± 3.0/min. greater after smoking in the fasting state than after the meal, and the difference is statistically almost significant ($p < 0.05$). The maximum pulse rate was observed at the end of smoking.

The systolic and diastolic blood pressures rose also a little more in the control experiments than in those with food intake. The difference was 4.4 ± 1.8 mm Hg in systolic and 2.3 ± 1.0 mm Hg in diastolic pressure, and both rises are just significant ($p < 0.05$). Also here the top values were measured in the end phase of smoking.

The skin temperature in fingers diminished $3.6° \pm 0.8$°C less in

smoking experiments after the meal than in the fasting state. The difference is highly significant ($p < 0.001$), although the vasodilatating effect of food itself is taken into consideration. After the meal the lowest temperatures were observed ca 10 minutes after the beginning of smoking, while in controls quite a slight decrease continued also later, up to the end of observation time.

The conclusion can be drawn from these experiments that certain cardiovascular responses to cigarette smoking, especially the reduction in skin temperature showing its blood flow, are weakened immediately after food intake.

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FROM THE INSTITUTE OF OCCUPATIONAL HEALTH, HELSINKI

URINE FLOW AND WATER BALANCE IN THE SAUNA-BATH

by

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It has been observed that the urine flow of hydrated dogs diminishes, if the animals are kept at a moderately elevated ambient temperature (3). This reduction of the urine flow does not occur in animals with experimental diabetes insipidus (3). The occurrence of a rise of the osmotic pressure of blood — which is an established stimulus for the release of the antidiuretic hormone (14) — is unlikely under the conditions of the experiment, since the dog does not loose water appreciably through evaporation under those conditions. Therefore the antidiuretic response is to be ascribed to a direct effect of the raised temperature on the anterior hypothalamus, which stimulates a release of the antidiuretic hormone from the posterior pituitary (3). An antidiuretic response, which outlasts the actual exposure by half an hour, has been described also in another »non-sweating» species, in rats kept at + 75°C for 5 min. (5).

In man, an exposure to heat also causes a reduction of the urine flow (15). In principle, this may be due to a decrease of the glomerular filtration, to an increase of the tubular reabsorption, or both. Exposures to moderate heat have been shown to cause a decrease of the glomerular filtration rate (2, 9, 10). On the other hand, antidiuretic substance has also been demonstrated in human urine following exposure to moderate heat for 90 min. (6). Thus, both glomerular and tubular mechanisms may be operative.

In the latter study, antidiuretic substance appeared in the urine only after the development of a negative water balance. Thus, the secretion of the antidiuretic substance may have been due to the rise of the osmotic pressure of blood and independent of the heat *per se*. In the present study, an attempt was made to prevent the development of a negative water balance, by using a short exposure to intense heat in the Finnish Sauna-bath, while water was being absorbed from the intestine at the same time. Thus, it was hoped to study the effect of heat without concomitant haemoconcentration. Moreover, by using the Finnish bath and Finnish subjects, for whom such an exposure to heat belongs to the weekly routine, an interference of the urine flow by emotional factors was considered minimal (12, 5).

METHODS

The subjects were 8 healthy adult males with body weights varying from 68 to 95 kg. The experiments were made after a fast of ca. 12 hours. In order to bring the subjects to a standard state of hydration before the experiment, they were given 0.5 per cent of body weight tap water 90 min. before the start of the experiment. In the hydration experiments, 1 per cent of body weight tap water was given as a second dose at the beginning of the experiment (= 0 min.).

At the beginning of the experiment the bladder was emptied, and after this urine was voided and its volume measured every 15 min. during two hours. Four blood samples were taken from the cubital veins: at 0 min. — at 30 min. (immediately before the bath) — at 45 min. (immediately after the bath) — at 75 min.

The subjects stayed in the bath for 15 min., the period from 30 to 45 min. from the beginning of the experiment. The dry bulb temperature varied from + 77 to 92°C, and the wet bulb from +40 to 45°C. Experiments with and without hydration were made alternatingly, one of the subjects being hydrated and another without hydration, in order to nullify any effects of variations in the bath temperature.

The coagulation of the blood samples was prevented with heparin. An aliquot was used for the photoelectric determination of the haemoglobin concentration (as acid haematin), and the remainder

was centrifuged without delay. The plasma chloride concentration was then determined titrimetrically (4, 13).

Sweat excreted during the exposure to heat was collected into a plastic arm bag. The sweat chloride concentration was determined by using the same method as with plasma, and the Na and K concentrations with an internal standard flame photometer (8). The volume of sweat collected in the arm bag was measured by draining it into a measuring cylinder. In order to get additional information on the sweat loss, the subjects were also weighed before and after the stay in the sauna-bath.

The following types of experiments were performed:

A. Control, with prehydration only (8 experiments).
B. Sauna-bath, with prehydration only (8 exp.).
C. Control, with prehydration and hydration (8 exp.).
D. Sauna-bath, with prehydration and hydration (8 exp.).

Four additional experiments were made with a hydration dose of 2 per cent of body weight.

The statistical analysis was made with the aid of the t-test.

RESULTS

An exposure to heat in the Sauna-bath caused a *suppression of the water diuresis*. This is clearly shown in Fig. 1. The differences between the mean volumes of urine collected are significant for 1 hour after the bath, but not in the sample excreted during the stay in the bath itself.

When no hydrating dose of water was given, the urine flow in the Sauna-experiments did not differ significantly from that in the corresponding controls during any period during or after the sauna. Neither were the summed volumes collected after the Sauna (from 45 to 120 min.) significantly different.

The exposure to heat was sufficient to bring the urine flow after hydration to quite the same level as in the experiments without hydration, and the urine flow of the hydrated subjects did not differ significantly from that of non-hydrated subjects after the Sauna; the same was true of the summed volumes collected after the bath.

On the other hand, the subjects exposed to heat without hydra-

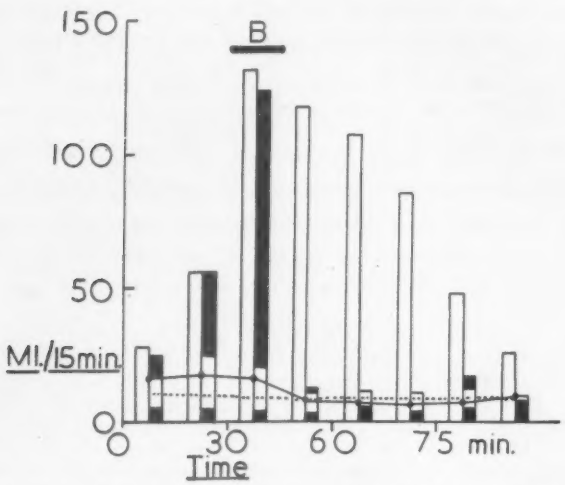

Fig. 1. — Mean urine flow in hydrated controls (white columns), in Sauna-experiments with hydration (black columns), in non-hydrated controls (dotted line), and in Sauna-experiments without hydration (solid line). B = exposure to heat.

TABLE 1

MEAN LOSS OF BODY WEIGHT AND MEAN VOLUME OF SWEAT COLLECTED FROM THE ARM BAG. (± STANDARD DEVIATION)

	Weight Loss, kg.	Volume of Sweat, ml.
With hydration	0.51±0.12	17.8±7.9
Without »	0.50±0.11	17.9±8.4

tion losed as much sweat as those hydrated, both as measured from the amount in the arm bag and as judged from the loss of weight (Table 1). Therefore, the urine flow of these groups after the bath was equal, in spite of a marked difference in the water load.

Fig. 2 shows the mean changes of the *plasma chloride* concentration in each group, and Fig. 3 the corresponding graphs for *haemoglobin*. When the changes between two successive samples are calculated, it is observed that during the *first half-hour* after hydration both the plasma chloride and the haemoglobin levels significantly fell (Table 2). With haemoglobin, the fall was relatively more marked.

During the *stay in the bath*, the plasma chloride level rose, as

TABLE 2

MEAN CHANGES OF PLASMA CHLORIDE (MILLIEQUIVALENTS PER LITRE) AND HAEMOGLOBIN (GM. PER 100 ML.) (± STANDARD DEVIATION) AND THEIR SIGNIFICANCE IF ABOVE THE P = 0.05 LEVEL

	Before Bath		During Bath		After Bath	
	Cl	Hb	Cl	Hb	Cl	Hb
Control without hydration	−0.8±1.2	−0.09±0.15	−1.1±2.6	−0.39±0.48 $p<0.05$	+1.2±2.2	−0.20±0.40
Sauna without hydration	−0.8±1.3	−0.02±0.16	+1.2±0.8 $p<0.01$	−0.02±0.44	+0.4±1.3	−0.20±0.45
Control with hydration	−1.3±1.6 $p<0.05$	−0.83±0.73 $p<0.05$	−0.2±0.4	+0.04±0.41	+1.1±0.9 $p<0.05$	+0.16±0.37
Sauna with hydration	−2.4±1.0 $p<0.001$	−0.92±0.55 $p<0.01$	+3.5±2.6 $p<0.01$	+0.67±1.03	−0.3±1.5	−0.47±0.34 $p<0.01$

was expected. However, the changes of the haemoglobin level during that period were variable and did not attain statistical significance.

After the bath, the haemoglobin level of the hydrated subjects further fell, but with chloride the corresponding change was slight and statistically insignificant. The plasma chloride of the hydrated controls, on the other hand, tended to rise back to the pre-experimental level.

The haemoglobin level had a tendency to fall in the controls

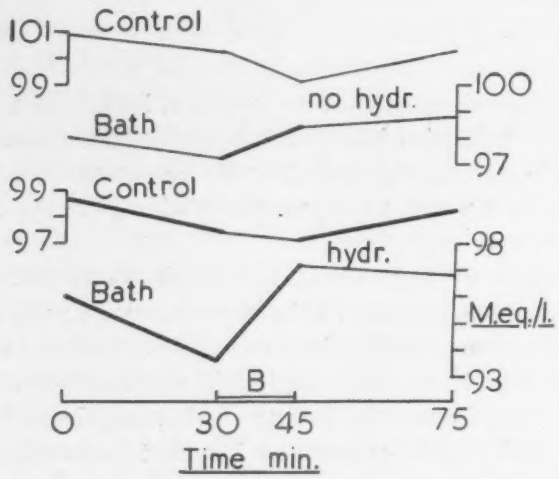

Fig. 2. — Mean plasma chloride level. Changes significant at least at the $p < 0.05$ level are drawn thick. B = exposure to heat.

without hydration throughout the period of the experiment, which change attained statistical significance. However, acceptance of controls as the »basis line» for the others will not materially change the type of the results, as seen in Fig. 2 and 3.

As mentioned above, the volumes of sweat secreted were equal after hydration and without it. Similarly, the concentrations of Na, K, and Cl showed no significant differences between these two groups (Table 3). The ratio Na/K did not either manifest any

TABLE 3

MEAN SODIUM, POTASSIUM, AND CHLORIDE CONCENTRATION (MILLIEQUIVALENT PER LITRE) AND NA/K RATIO IN THERMAL SWEAT (± STANDARD DEVIATION) WITH AND WITHOUT HYDRATION. n = NUMBER OF DETERMINATIONS

	Na	K	Cl	Na/K
With hydration	55.2 ± 6.4	13.7 ± 6.1	54.1 ± 15.9	4.61 ± 1.79
n:	7	7	8	7
Without hydration	61.0 ± 17.7	12.2 ± 5.7	54.8 ± 19.2	5.83 ± 2.44
n:	7	7	8	7
Significance of difference	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$

regular difference between the two series. These observations conform to previous studies (1).

Since the sweat loss in the bath was almost of the same magnitude as the amount of water ingested, the water load after sweating was rather small. Therefore, four experiments were made with a double amount of water, but similar exposure to heat. In these experiments, an antidiuretic response also occurred, but the urine flow did not reach equally low values, and it increased again more markedly than in the series with the smaller hydration dose.

In these four experiments, the changes of the haemoglobin level were similar to those shown in Fig. 3. With chloride the direction of the changes in three of the subjects corresponded with those shown in Fig. 2 (with varying changes during the last period), but in one, the chloride level remained absolutely steady. Nevertheless an antidiuretic response was observed (Fig. 4). In none of these subjects did the chloride level after the bath exceed the starting level.

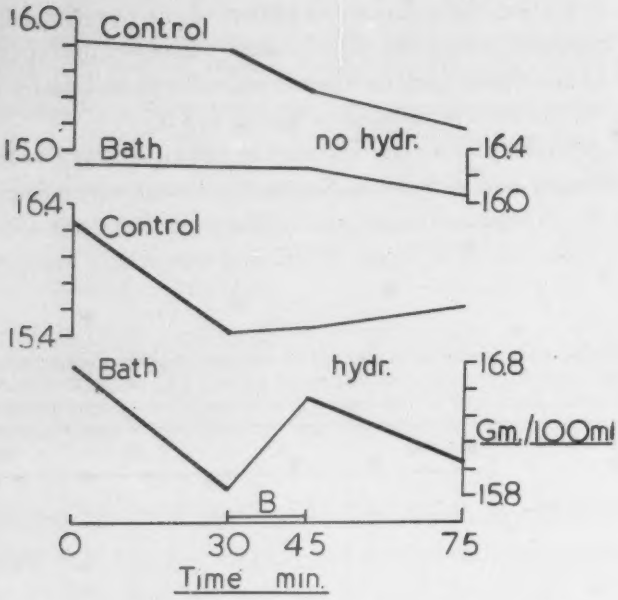

Fig. 3. — Mean haemoglobin level. Symbols as in Fig. 2.

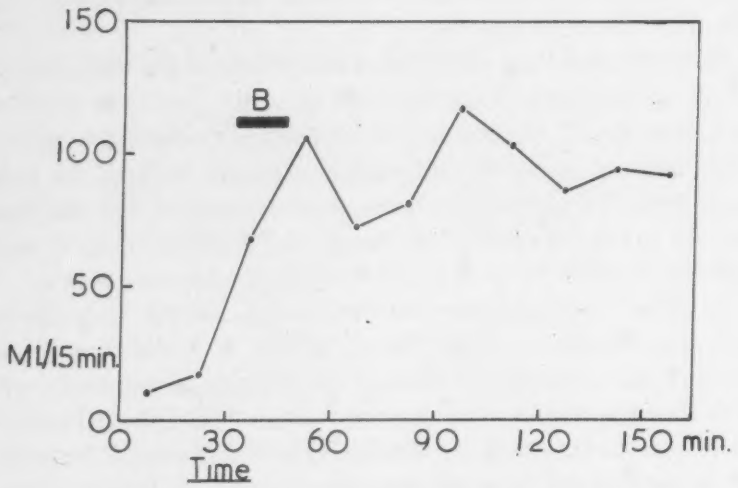

Fig. 4. — Urine flow in an experiment after ingestion of 1,500 ml. water at time O. In spite of steady plasma chloride level, a marked antidiuretic response ensued.

DISCUSSION

An exposure to the heat in the Sauna-bath regularly caused an antidiuretic response in hydrated subjects. The antidiuresis did not become evident during the 15 minutes the subjects stayed in the heat, but it was maximal — as compared with the urine flow in the corresponding controls — during the immediately succeeding 15 min. period and remained lower than the control rate for 75 min. after the bath with a hydration of 1 per cent of body weight. With the higher hydration (2 per cent of body weight) the changes were basically similar. In other words, there is a latency of the order of 15 min. between the beginning of the exposure and the antidiuresis, and when once elicited, the antidiuretic response long outlasts the stimulus. When antidiuretic hormone is injected intravenously, the maximum antidiuretic effect has a latency of 11 to 13 minutes (14), and the passing of the effect of even minute amounts requires at least half an hour. Thus, the observed antidiuresis following exposure to heat resembles in its time relations that caused by the antidiuretic hormone.

After drinking water, the water balance of the body may be disturbed in three ways. First, isotonic fluid with a chloride concentration exceeding that of plasma may be secreted by the glands of the stomach. This can be expected to lead to a concentration of haemoglobin and to dilution of the plasma chloride level. Second, salt, mainly sodium chloride, may enter the gastro-intestinal tract, due to osmotic forces, without the secretion of corresponding volume of water. This tends to cause a concentration of plasma chloride, without — at least as marked — a concentration of haemoglobin. Third, the absorption of water tends to dilute both the haemoglobin and the chloride levels. However, the changes in plasma chloride are rapidly distributed over the chloride space of the body, whereas haemoglobin has no corresponding »buffer space», and the resulting changes may be expected to be more marked with haemoglobin than with chloride.

Heat, on the other hand, causes the loss of hypotonic fluid, sweat, and may thus be expected to raise both the haemoglobin and plasma chloride levels; again, the changes of the haemoglobin may be expected to be more marked than those of plasma chloride.

When the changes of haemoglobin and chloride between two

successive samples were correlated, the correlation coefficient was $r = +0.851$. Thus, the observed changes of the Hb and levels were to a great extent parallel.

Of the factors listed above, the dilution of the haemoglobin and chloride levels due to the absorption of water was clearly predominant during the first half-hour after hydration. During the exposure to heat, the plasma chloride concentration rose, but with haemoglobin the changes were variable. In the hydration experiments this behaviour may have been due to the competitive effects of the loss of sweat and simultaneous absorption of water — which had already more or less attained osmotic equilibrium with blood — from the gastrointestinal tract. Thus the net result would be a concentration of chloride with variable changes in the haemoglobin concentration. The lack of a rise in the haemoglobin level during an exposure to heat without hydration is less easy to understand.

In the controls without hydration, the haemoglobin level fell progressively during the experiment, and the fall reached statistical significance at the $p < 0.05$ level. This fall must be understood as an expression of diurnal variations, since a significant diurnal fall of the Hb level is known to occur from early morning to afternoon (11), which fits the present data. The blood chloride, on the other hand, shows no regular diurnal cycle (11).

The chloride level, which may be considered as an indicator of changes in the osmotic pressure of the blood, rose during the exposure to heat. In some experiments this rise fully compensated for the preceding fall due to hydration, but in others it did not bring the chloride to the starting level. Nevertheless, in all hydration experiments there was a marked antidiuretic response after the bath. Therefore, the stimulus for the antidiuresis evidently cannot have been an absolute rise of the plasma osmotic pressure. This still leaves the possibility that a relative rise of the osmotic pressure of the blood has contributed to the observed response. However, even in such an experiment, in which the chloride level did not fall (Fig. 4), there was a definite antidiuretic response. Thus, the present series makes it appear that an antidiuretic response in the human subject exposed to heat is due both to some »direct» effects of heat and to the haemoconcentration caused by loss of sweat. Further a relative haemoconcentration may be sufficient to contribute to the stimulus.

Though the present results can be interpreted on the basis of analogy with an effect of the antidiuretic hormone on tubular processes, this does not, of course, exclude other effects of heat, direct, nervous or humoral, on the kidney (cf. 2, 7, 9, 10).

The occurrence of an intense antidiuretic response after an exposure to heat, independently of the absolute level of the osmotic pressure of blood, opens the possibility for the development of water intoxication. Whether a fall of the osmotic pressure of the blood and symptoms of water intoxication will ensue, depends on the amount of water in the gastrointestinal tract at the onset of the antidiuresis, and, on the other hand, on the sweat loss. A bath soon after the ingestion of water — or water-containing food — will favour the development of this condition, likewise as an intense bath of short duration, after which sweating is being stopped with rapid cooling of the body.

SUMMARY

An exposure to intense heat in the form of the Finnish Sauna-bath was shown to cause a prolonged suppression of water diuresis after the bath. An antidiuretic response occurred independently, whether the plasma chloride level rose above the starting level or not during the exposure to heat.

The conclusion is drawn that an exposure to heat may lead either to haemoconcentration, as generally expected, or to haemodilution, if water is being absorbed after a short and intense exposure to heat.

The sweat loss and the sweat Na, K and Cl concentrations as well as the sweat Na/K ratio were unaffected by the ingestion of 1 per cent of body weight of water 30 min. before the 15 min. exposure to heat.

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